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Novel Changes in Glycosylation of Serum Apo-J in Patients with Hepatocellular Carcinoma

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Abstract

Background—Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and the occurrence of HCC has more than doubled in the United States (USA) in the last decade. Early detection is considered key to reducing the mortality of HCC.

Methods—Using two-dimensional gel electrophoresis and high performance liquid chromatography (HPLC) we have analyzed the glycosylation of Apo-J from healthy controls, patients with liver cirrhosis or with hepatocellular carcinoma.

Results—Apo-J in the serum from patients with HCC had decreased levels of (β-1,4) triantennary N-linked glycan compared with the healthy controls or patients with liver cirrhosis. We analyzed this change in an independent cohort of 76 patients with HCC, 32 with cirrhosis, and 43 infected with hepatitis C virus using the *Datura stramonium* lectin (DSL), which binds to (β-1,4) triantennary N-linked glycan. The level of DSL-reactive Apo-J allowed us to differentiate HCC from cirrhosis with an area under the receiver operating characteristic curve (AUROC) of 0.852. When Apo-J was combined with other serum biomarkers such as alpha-fetoprotein or fucosylated kininogen using a multivariate logistic regression model, the AUROC increased to 0.944, a value much greater than that observed with AFP alone (AUROC of 0.765).

Conclusions—The glycosylation of Apo-J is a useful marker when used alone or in combination with other markers for the early detection of HCC.

Impact—The potential use of a combination of alpha-fetoprotein, DSL-reactive Apo-J, and fucosylated kininogen as a biomarker of HCC would have great value in the management of patients with liver disease.

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Introduction

Infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is the major etiology of hepatocellular cancer (HCC) (1-4). Both HBV and HCV cause acute and chronic liver infections, and most chronically infected individuals remain asymptomatic for many years (5). A total of 10% to 40% of all chronic HBV carriers eventually develop liver cancer, and it is estimated that more than one million people worldwide die of HBV/HCV-associated liver cancer (2,6,7). Indeed, HBV and HCV infections are associated with over 80% of all HCC cases worldwide and can be as high as 96% in regions where these viruses are endemic (3).

The progression from liver disease to liver cancer is monitored primarily by serum levels of oncofetal glycoprotein, alpha-fetoprotein (AFP), or the core fucosylated glycoform of AFP (AFP-L3). However, AFP can be produced under many circumstances, including in association with other liver diseases (8-10), and it is not present in all patients with HCC (11). Therefore, the use of AFP as a primary screen for HCC has been questioned (12), and more sensitive serum biomarkers for HCC are desired.

Using various proteomic methods to look for biomarkers for HCC, we identified changes in the levels of glycan in total serum and in serum depleted of IgG or of the major acute phase proteins (13-15). In this study, we expanded our search by analyzing the level of glycosylation of an individual protein, Apo-J, as a function of HCC. Apo J, also known as clusterin, is a secreted glycoprotein with a yet unknown function that we (13,14,16) and others (17-21) showed to be associated with HCC. Apo J contains 7 potential sites of glycosylation and is generally found in up to 6 isoforms with differing molecular weights and charges (21). Using the N-linked glycan analysis, we compared Apo J levels in healthy subjects with those in patients with HCC and found a specific decrease in the amount of (β -1,4) triantennary N-linked glycan in patients with HCC. We analyzed this change in glycosylation in two independent cohorts comprising over 200 patients, and determined the ability of this marker to differentiate HCC from liver disease.

Materials and Methods

Patients

We obtained serum samples from the University of Michigan ($n = 60$: HCC = 20, cirrhosis = 20, healthy controls = 20), Saint Louis University School of Medicine ($n = 151$: HCC = 76, cirrhosis = 32, HCV infected = 43), and the California Pacific Medical Center ($n = 60$: HCC = 10, liver disease = 50). We obtained approval for the study protocol from the appropriate institutional review boards, and written informed consent was obtained from each subject. Details on patients from the University of Michigan and St. Louis University School of Medicine are provided elsewhere (22). We obtained demographic and clinical information for each patient from the California Pacific Medical Center.

A blood sample collected from each subject was placed in a serum separator tube and spun for 2 hours; the serum was stored at -80°C until testing. The diagnosis of HCC was made by histopathological analysis, including all T1 lesions. If histopathological samples were not available, the diagnosis was made from the results of 2 imaging modalities (ultrasound, magnetic resonance imaging, or computed tomography) showing a vascular-enhancing mass > 2 cm. Diagnosis of cirrhosis was based on histological analysis of liver tissue or on clinical, laboratory, and imaging evidence of hepatic decompensation or portal hypertension.

Two-dimensional gel electrophoresis

A total of 7 μ l of serum was diluted in 330 μ l of solubilization buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithiothreitol (DTT), 5 mM tributylphosphine, and a 0.4% mixture of carrier ampholytes (Servalyt pH 2-4, pH 3-10, pH 9-1, 1:2:1). Samples were vortexed periodically for 1 hour and applied to a 17-cm pH 3 to 6 non linear (NL) immobilized pH gradient strip (Bio-Rad Laboratories, Hercules, CA, USA). Gel rehydration was carried out for 14 hours at 50 V and focused using the Protean (Bio-Rad) isoelectric focusing system. After the focusing, gel strips were first reduced then alkylated in 6 M urea, 2% SDS, 30% glycerol, 50 mM Tris, pH 6.8, and either 30 mM DTT or 75 mM iodoacetamide for 10 min each. The second dimension was resolved with an 8% to 18% acrylamide–0.8% piperazine diacrylamide (PDA) gradient gel on a Protean II xi cell (Bio-Rad) with the running conditions set to 20 mA/gel for 20 min and 40 mA/gel for 4 hours. Gels were fixed and stained with colloidal Coomassie. Gels were imaged using the Odyssey Infrared Imaging System (Li-Cor Biosystems, Lincoln, Nebraska) and analyzed using NonLinear Dynamics Progenesis Workstation gel imaging software (Nonlinear Dynamics, Durham, NC, USA).

Glycan analysis

The gel spots were excised and denatured with DTT at 100°C for 5 min, allowed to cool, and alkylated in the dark for 30 min with iodoacetamide. The gel plugs were fixed in a solution of 30% ethanol and 7% acetic acid for 1 hour. The gel plugs were washed by dehydration in acetonitrile (ACN), rehydration in 20 mM ammonium bicarbonate, and dehydration with ACN, then dried in a speed-vac. Peptide:N-glycosidase F (PNGase F) was diluted with 20 mM ammonium bicarbonate pH 7 and allowed to adsorb into the gel plug. The gel plug was then covered with the same solution and allowed to incubate overnight at 37°C. The glycans were eluted from the gel plug by sonication in Milli-Q water 3 times; the elutant pooled was dried down and labeled with a 2-aminobenzamide dye (Ludger, Oxford, UK) according to the manufacturer's instructions. Excess dye was removed using paper chromatography and the glycans were passed through a 0.22- μ m syringe filter. Fluorescently labeled glycans were subsequently analyzed by high-performance liquid chromatography (HPLC) using a TSK-Gel Amide 80 column. The mobile phase consisted of solvent A (50 mM ammonium formate, pH 4.4) and solvent B (ACN). The gradient used was as follows: linear gradient from 20% to 58% solvent A at 0.4 mL/minute for 152 min followed by a linear gradient from 58% to 100% solvent A for the next 3 min. The flow rate was increased to 1.0 mL/minute, and the column was washed in 100% solvent A for 5 min. Following the wash step, the column was equilibrated in 20% solvent A for 22 min in preparation for the next sample run. HPLC analysis was performed using the Waters Alliance HPLC System, complemented with a Waters fluorescence detector, and quantified using the Millennium Chromatography Manager (Waters Corporation, Milford, MA). Glycan structures were identified by calculating the glucose unit value and exoglycosidase digestion, as previously described (23).

Liquid chromatography/mass spectrometry/mass spectrometry

Following glycan preparation, the deglycosylated gel plug was dehydrated in ACN and dried. Trypsin-Gold (Promega, MD), diluted to 20 μ g/mL according to the manufacturer's recommendations, was absorbed into the dried gel plug on ice. Following rehydration, the gel plugs were covered with 25 mM ammonium bicarbonate buffer and incubated at 37°C overnight. Peptides were extracted twice using 60% ACN, 0.1% trifluoroacetic acid with sonication and once with 100% ACN. The extractions were pooled together, dried, and resuspended in 5% ACN, 0.1% trifluoroacetic acid. The peptides were concentrated and desalted using ZipTip C18 (Millipore, Bedford, MA, USA). Peptides were analyzed using a Dionex UltiMate 3000 Nano liquid chromatography system (Dionex, Sunnyvale, CA)

coupled with a Finnigan LTQ mass spectrometer. Peptides were separated online using a 75- μ m ID x 15-cm C18 analytical column using 2% ACN/0.1% formic acid (buffer A) and 90% ACN/0.1% formic acid (buffer B). Following the initial wash with 95% buffer A for 10 min, peptides were eluted from the column during a 90-min linear gradient of 5% to 60% of buffer B at a flow rate of 250 η L/min directly into a linear trap quadrupole ion trap mass spectrometer.

Lectin–fluoropore-linked immunosorbent assay

Antibody (1 μ g/well in phosphate buffered saline [PBS], pH 7.4) from Sigma-Aldrich (St. Louis, MO), was added to the plate and, following incubation overnight at 4°C, was washed with phosphate buffered saline/0.1% Tween 20 pH 7.4 and blocked overnight with 1 $\times$ Carbo-Free blocking buffer (Vector Laboratories, Burlingame, CA). For analysis, 1 μ l of serum was diluted in 49 μ l PBS with 0.1% Tween-20 and was incubated at room temperature for 2 hours. Subsequently, plates were washed 5 times with 0.1% Tween 20/PBS pH 7.4, then washed three times with lectin incubation buffer (25 mM Hepes, 1mM MnCl₂, 0.1% Tween 20) before triantennary glycan was detected with a biotin-conjugated *Datura stramonium* lectin (DSL) (Vector Laboratories). Bound lectin was detected using IRDye 800 conjugated streptavidin; signal intensity was measured using the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, Nebraska). In all cases, signal intensity was compared to signals detected with commercially purchased human serum (Sigma-Aldrich).

Statistical analysis

Descriptive statistics for patients were compared by scatter plots that included the outliers. All values were reported as mean values $\pm$ standard deviation unless otherwise stated. Because the data did not follow a typical Gaussian distribution, a nonparametric test (two-tailed, 95% confidence, Mann-Whitney Test) was used to determine statistical differences between groups. To determine the optimal cutoff value for each marker, the receiver operating characteristic (ROC) curves were constructed using all possible cutoffs for each assay. The area under the ROC (AUROC) curves were constructed and compared as described previously. A two-tailed *P* value of 0.05 was used to determine statistical significance. All descriptive analyses were performed using a GraphPad Prism (San Diego, CA). For combinatorial analysis, AFP values were log transformed to bring the values of all markers into a similar scale, and a centering and scaling approach was taken to normalize data before analysis using a multivariate logistic regression method, using the R package, version 2.8.1.

Results

Glycosylation of Apo-J is altered with the development of liver cancer

Samples of pooled sera from healthy patients (*n* = 20), patients with liver cirrhosis (*n* = 20), and patients with HCC with a background of cirrhosis (*n* = 20) were resolved via two-dimensional gel electrophoresis (2-DE) (Table 1). Fig. 1A shows the results of representative 2-DE of normal human serum and Figs. 1B, 1C, and 1D show the Apo-J alpha- and beta-chain isoforms from the three patient groups. The Apo-J concentrations in each patient group were comparable (Figs. 1B-1D) and fell within normal serum concentrations. In the control sample, the level of Apo-J was 100 μ g/mL ($\pm$ 1.4 μ g/mL), whereas in the patients with cirrhosis and HCC, the level of Apo-J was 112 μ g/mL ($\pm$ 1.4 μ g/mL) and 110 μ g/mL ($\pm$ 1.4 μ g/mL), respectively. Each of the 5 APO-J isoforms was excised and combined, and the N-linked glycans were analyzed by normal phase HPLC following LC MS/MS analysis to confirm protein identity and purity (data not shown).

N-linked glycans attached to Apo-J were removed using peptide:N-glycosidase F and labeled with a fluorescent dye before analysis of the N-linked glycans via sequential exoglycosidase digestion (24-26). Fig. 2 shows the simplified desialylated glycoprofile of Apo-J for each patient group following treatment with neuraminidase (*Arthrobacter ureafaciens*). Two peaks that were altered in the HCC sample are indicated with an asterisk in Fig. 2A. Sequential exoglycosidase digestion (Supplementary Fig. S1) showed these peaks to be a simple biantennary glycan (A2G2) and a (β -1,4) triantennary N-linked glycan (A3G3). In the control and cirrhosis groups, the biantennary glycan represented 42% (± 4.2) and 45% (± 4.9) of the total glycan pool, respectively (Fig. 2B). In the HCC group, the biantennary glycan represented 51% (± 7.8) of the total glycan pool. Fig. 2B also shows the level of the A3G3 glycan in the three patient groups. The level of the A3G3 glycan represented 37% (± 4.1) of the total glycan pool from the control patients and 32% (± 4.9) from the patients with cirrhosis, whereas it represented 21% (3.7%) of the total glycan pool in the HCC group.

Analysis of (β -1,4)-linked A3G3 Apo-J by lectin-fluorochrome-linked assay in a cohort of 151 patients

To further examine if the changes in glycosylation observed in the pooled samples could be observed on individual samples and in an independent cohort, we used a lectin-fluorochrome-linked immunoassay (FLISA). Because no lectins that can bind biantennary glycans are available, we used DSL, which can recognize (β -1,4)-linked triantennary glycan. In this assay, Apo-J was captured using a monoclonal antibody, and the level of A3G3 glycan was determined using DSL. We noted that the lectin-FLISA detects the amount of triantennary glycan present on an equal quantity of captured molecules from each patient sample and is performed in a manner that is independent of the total amount of protein in a sample from any given patient. Thus, even if protein levels are different in different individuals, this assay measures only the relative proportion of altered glycoprotein. This method was first tested on the pooled samples used in Figs. 1 and 2; the results are shown in Fig. 2C. As these results show, we observed a decrease in the level of A3G3 glycan, similar to that observed when using glycan sequencing of Apo-J. This result was further analyzed in a patient cohort consisting of 151 patients using the lectin-FLISA (Table 1). In this cohort, 43 patients were infected with HCV; 32 patients were infected with HCV and had liver cirrhosis; and 76 patients had liver cancer (Table 1). Fig. 3A shows a scatter plot of the level of DSL-reactive Apo-J in the three patient groups. Values are given as fold increase over the level in commercially purchased “normal” sera. We found a clear statistical difference between the HCC group and the cirrhotic group ($P < 0.0001$) but not between the HCV-infected and the cirrhosis group ($P = 0.6184$). The mean level of DSL-reactive Apo-J was 0.91-fold (± 0.30) in the HCC group, 1.2-fold (± 0.16) in the cirrhosis group, and 1.2-fold in the HCV infected group (± 0.20). Surprisingly, we did not see any difference in the mean level of AAL-reactive Apo-J in HCC patients at different stages of HCC (data not shown). Patients with stage 1 HCC, defined as a single lesion of less than 2 cm (11), had a 0.855-fold (± 0.14) level of DSL-reactive Apo-J whereas patients with stage 4 HCC (those with multiple lesions > 6 cm in diameter) had a mean of 0.779 (± 0.13), which was not different from that observed in stage 1 patients ($P = 0.4204$). In this cohort, DSL-reactive Apo-J distinguished between HCC and cirrhosis with an AUROC of 0.852. This result was greater than the results obtained with AFP in this cohort (0.7646). In this cohort, AFP had a mean value of 60.1 ng/mL in the HCC patients and 10.4 ng/mL in the cirrhotic patients.

Combinatorial analysis of DSL-reactive Apo-J with AFP and fucosylated kininogen in the detection of HCC

Fig. 4A shows a perspective plot of the relative levels of DSL-reactive Apo-J, AFP, and another marker previously examined in this cohort, fucosylated kininogen (22). Most HCC

patients (represented by solid red dots) were found in the lower back corner of the plot, indicating that they had high levels of AFP and fucosylated kininogen but low levels of DSL-reactive Apo-J. In contrast, the cirrhotic samples (represented by open circles) cluster in the bottom left hand corner of the plot, indicative of low levels of the individual markers.

When DSL-reactive Apo-J was combined with AFP using a multivariate logistic regression analysis, the performance was improved to 0.9107. Similarly, as Fig. 4B shows, when combined with another potential marker, fucosylated kininogen, the AUROC was 0.9139. When all three markers were included (Fig. 4B) in a multivariate logistic regression model, the AUROC increased to 0.944.

Analysis of the combined markers in a second independent cohort

The performance of DSL-reactive Apo-J alone and in combination with fucosylated kininogen was tested in an independent cohort of 60 patients, 10 of whom had HCC and 50 of whom had liver disease (Table 1). In this cohort, using the algorithm determined in the first sample set, the AUROC of DSL-reactive Apo-J and fucosylated kininogen that differentiated HCC from liver disease was 0.9129, with an optimal sensitivity of 87% and a specificity of 96%, similar to what was obtained in the initial training set.

Discussion

Serum levels of Apo-J have been reported to be associated with both the presence of HCC and the metastatic potential of the cancer (17,19,21). In addition, we previously identified Apo-J as being part of the serum fucosylated proteome in patients with HCC, which suggested a difference in glycosylation of Apo-J. To further examine the glycosylation of Apo-J, we performed N-linked glycan analysis of Apo-J following separation using 2-DE. Serum Apo-J from patients with HCC had less triantennary N-linked glycan compared with Apo-J from healthy controls or patients with liver cirrhosis. The change in glycosylation was analyzed using a lectin ELISA in which a saturating amount of Apo-J was captured and the relative level of triantennary N-linked glycan was determined using a lectin (DSL) specific for this glycan structure. Like the N-linked glycan data, the lectin-ELISA data confirmed the decrease in triantennary glycan in a larger sample set. The ability of DSL-reactive Apo-J to differentiate cirrhosis from HCC was greater than that of AFP. When used in combination with AFP through multivariate analysis, DSL-reactive Apo-J could distinguish cirrhosis from cancer with an AUROC of 0.9107, with an optimal sensitivity of 86% and a specificity of 95%. Analysis of the multivariate model in a second, independent patient cohort was used to confirm the multivariate analysis, and a similar AUROC was observed (0.9139).

Although alterations in fucosylation have been observed on serum proteins as a function of HCC, this report describes a decrease in triantennary branching with the development of liver cancer. It is important to note that the decrease in triantennary glycan associated with Apo-J was also correlated with an increase in the simple biantennary glycan associated with Apo-J from HCC patients. This result suggests that a decrease may occur in the level of the N-acetylglucosaminyltransferase IV (GnT-IV) enzyme, which can extend the biantennary glycan chain to the triantennary form (27).

Apo-J has been implicated as a biomarker of HCC. Previous reports have indicated that serum levels of Apo-J decreased in patients with cirrhosis (compared to healthy controls) but increased in those with HCC. In our limited analysis of patients with cirrhosis and HCC, we did not see any statistical difference in the level of Apo-J in the HCC group compared with the non-HCC groups (data not shown). It should be noted that our plate-based approach for the detection of Apo-J with triantennary glycan was performed under saturating conditions and examined equal amounts of captured Apo-J.

In summary, we have discovered a novel glycan modification on Apo-J and developed a lectin-FLISA to measure this change. DSL-reactive Apo-J could distinguish HCC from cirrhosis to the same degree as AFP. However, when used in combination with AFP or AFP and another potential marker, fucosylated kininogen, DSL-reactive Apo-J performed better than AFP alone. We postulate that these markers could be used to supplement AFP as a general screen in those patients at high risk for HCC development, either alone or in combination with ultrasound, which is the current practice. These markers may also be useful in monitoring HCC patients following treatment as well as identifying those patients with recurrence.

These data must be confirmed in larger cohorts of patients to determine if these markers are true indicators of early HCC and to compare the accuracy of the data with data of AFP in patients of diverse gender, ethnicity, and etiologies of liver disease and to determine its role in HCC surveillance. Future studies should also test the benefit of combinatorial analysis with other potential markers of HCC, such as des-gamma-carboxy prothrombin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Two-dimensional gel purification of Apo-J. Sera from pools of healthy controls (A, B) and patients with cirrhosis (C) and cancer (D) were focused using IPGPhor 3-7 NL first-dimension strips followed by SDS-PAGE separation on 8% to 18% acrylamide gels.

Figure 2.

The desialylated N-linked glycan profile of Apo-J from normal controls (top), cirrhotic (middle), or HCC patients (bottom). A, The major peaks that are altered are indicated with an asterisk and are (from left to right) a biantennary glycan (A2G2) and a triantennary N-linked glycan (A3G3). B, The percent of the A2G2 and A3G3 peaks in each of the three patient groups. For each patient group, individual pools were examined in triplicate by normal phase HPLC. C, Lectin-FLISA of DSL-reactive Apo-J on the same pooled samples on which glycan analysis was performed.

Figure 3.

Decrease in DSL-reactive Apo-J with the development of HCC. A, Scatter plot of the level of DSL-reactive Apo-J in patients with HCV, HCV-induced cirrhosis, and HCC. The solid line represents the mean value. The x-axis represents the patient group. The y-axis shows the fold change in DSL-reactive Apo-J compared with that in commercially purchased serum. Samples are as seen in Table 1. B, Receiver operator characteristic (ROC) curves for DSL-reactive Apo-J. The AUROC for DSL-reactive Apo-J was 0.852 at differentiating HCC from cirrhosis.

Figure 4.

A, Perspective plot of the relative levels of DSL-reactive Apo-J, fucosylated kininogen, and AFP. The solid circles represent individual HCC patients and open circles represent patients with cirrhosis. For the plot, log transformed values for each marker were used. B, Receiver operator characteristic (ROC) curves for Apo-J, fucosylated kininogen, AFP or a combination of all three markers between all HCC patients and cirrhosis. The AUROC for Apo-J is 0.855; 0.765 for AFP; 0.745 for fucosylated kininogen; and 0.944 for all three together.

Patient Data

Table 1

Source of Sample	University of Michigan			St. Louis University			California Pacific Medical Center	
	Healthy	Cirrhosis	HCC	HCV-infected	Cirrhosis	HCC	Liver Disease	HCC
Disease Diagnosis*								
Number	20	20	20	43	32	76	50	10
Etiology, % (HBV/HCV/crypt o/alcohol/other) [†]	N/A	5/48/20/18/9	11/51/24/10/4	N/A	N/A	14/52/6/20/8	20/60/20/00/00	30/20/40/10/0
Age, years ± SD	54, 3	58, 3	58.6, ±12	55±8	50±8	58.04 ±11	51±13	58±8
Gender, M:F, %	50:50	60/40	75/25	50:50	84:16	71:29	56:44	90:10
MELD score [‡]	N/A	9±2	10.3±4	N/A	N/A	11.8±5	NA	NA
Child class, A/B/C/ %or NA [§]	N/A	40/54/6/0	48/42/10/0	N/A	88:8:4	52/29/9/10	NA	NA
Tumor stage (1/2/3/4) %**	N/A	N/A	21/40/26/13	N/A	NA	26/48/12/14	NA	100/0/0/0

* HCC or cirrhosis was determined by MRI or by liver biopsy.

[†] Etiology: HBV, hepatitis B virus; HCV, hepatitis C virus; crypto, cryptogenic liver disease; alcohol, alcohol-induced liver disease; other, liver disease of unknown origin.

[‡] MELD: Model for end-stage liver disease.

[§] The percent of patients with each Child-Pugh score is given as a percentage in each group.

** Tumor staging was determined using the United Network of Organ Sharing-modified TNM staging system for HCC. The percent of patients within each stage is given.

Note: N/A, not available