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Total serum glycan analysis is superior to Lectin-FLISA for the early detection of hepatocellular carcinoma

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Abstract

Purpose—Hepatocellular carcinoma (HCC) is a primary cancer of the liver that is predominantly the result of infection with a hepatotropic virus such as hepatitis B virus (HBV) or hepatitis C virus (HCV). As liver cancer is often asymptomatic, the development of sensitive non-invasive biomarkers is needed for early detection and improved survival.

Experimental Design—We have previously identified alterations in the N-linked glycosylation of serum proteins with the development of HCC and identified many of the proteins that contained the altered glycosylation. In the current study, we compared the ability of the identified proteins to diagnose HCC with the total serum glycan analysis.

Results—Surprisingly, glycan analysis of total serum had the greatest ability to distinguish HCC from cirrhosis with an AUROC of 0.851, a sensitivity of 73% at a specificity of 88%. When total glycan sequencing was combined with alpha-fetoprotein (AFP), the sensitivity increased to 95% at a specificity of 90%.

Conclusion and clinical relevance—Changes in glycosylation as detected in whole serum could be used to diagnose HCC with greater sensitivity and specificity than that observed through the analysis of specific protein glycoforms or protein levels. Such an assay could have value in the management of those at risk for the development of HCC.

Keywords

Cirrhosis; glycomics; hepatitis B virus; hepatitis C virus; Hepatocellular carcinoma

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1. Introduction

Hepatocellular carcinoma (HCC), primarily caused by infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV), is one of the most common solid malignancies worldwide and the incidence in the United States (USA) is increasing [1-4]. The development of liver fibrosis and liver cirrhosis is recognized as the major risk factor for the development of HCC [3, 6, 7] and most cases of HCC occur in the background of cirrhosis. Although there are many causative agents for cirrhosis, chronic viral infections of the liver by HBV and/or HCV are among the most common etiologies.

The progression of liver disease into liver cancer is routinely monitored by the measurement of serum levels of the oncofetal glycoprotein, alpha-fetoprotein (AFP), which is thought to be produced by transformed liver cells; however, AFP can be produced under many circumstances, including other liver diseases and is not present in all those with HCC [8-10]. Hence the use of AFP as a primary screen for HCC has been questioned [6] and more sensitive serum biomarkers for HCC are desired.

Using fucose specific lectins to identify the proteins that become fucosylated with liver disease, we have identified more than 30 glycoproteins that contained increased fucosylation with HCC and/or cirrhosis[11] and have used these in plate-based assays to diagnose HCC[12-14]. However, these plate-based assays were hampered by the presence of heterophilic antibodies[15, 16] and potentially other lectin binding contaminants that theoretically could limit their use. In the current study, we directly compared N-linked glycan analysis of total (all) serum proteins to the lectin-FLISA or Lectin-Western of specific identified proteins in the detection of HCC. Surprisingly, a method of analysis of N-glycans derived from total serum, without any modification of serum, had the greatest ability to distinguish HCC from cirrhosis with an AUROC of 0.851 with a sensitivity of 73% at a specificity of 88%. In contrast, the examination of AAL reactive alpha-1-antitrypsin (A1AT) by immunoprecipitation lectin blotting (IP-Lectin blot) had an AUROC of 0.780 at differentiating HCC from cirrhosis. The potential use of total serum glycan analysis as a diagnostic tool for the detection of liver cancer in those infected with HBV and or HCV is discussed.

2 Material and Methods

2.1 Patient Samples

Serum samples were obtained from the University of Michigan under a study protocol approved by the University of Michigan's Institutional Review Board and written informed consent was obtained from each subject. Demographic and clinical information was obtained, and a blood sample was collected from each subject. Consecutive patients with HCC, and patients with cirrhosis that were age, gender, and race/ethnicity matched to the HCC patients were enrolled from the Liver Clinic during this period. The diagnosis of HCC was generally made by histopathology. However, if histopathology was not available, the diagnosis of HCC was made by two imaging modalities (ultrasound [US], magnetic resonance imaging [MRI], or computed tomography) showing a vascular enhancing mass > 2 cm) (5). Diagnosis of cirrhosis was based on liver histology or clinical, laboratory and

imaging evidence of hepatic decompensation or portal hypertension (15). Each of the patients with cirrhosis had a normal US and, if serum AFP was elevated a MRI of the liver within 3 months prior to enrollment and another one 6 months after enrollment that showed no liver mass. The cirrhotic controls were followed for a median of 12 months (range 7-18 months) after enrollment, and no one has developed HCC. Tumor staging was determined using the United Network of Organ Sharing-modified TNM staging system for HCC (16). Early HCC was defined as T1 (single lesion < 2 cm in diameter) and T2 (single lesion between 2 and 5 cm in diameter; or < 3 lesions each < 3 cm in diameter) lesions, which met criteria for liver transplantation in the United States. A 20-ml blood sample was drawn from each subject, spun, aliquoted, and serum stored at -80°C until testing. Blood samples were drawn prior to initiation of HCC treatment. AFP was tested using commercially available immunoassays utilizing enhanced chemiluminescence at the University of Michigan Hospital Clinical Diagnostic Laboratory.

2.2 N-linked Glycan analysis

N-linked Glycan analysis was performed on total serum after absorption of serum into 12% acrylamide gel plugs. Briefly, before use, acrylamide gel plugs were dehydrated in acetonitrile, rehydrated in 20mM ammonium bicarbonate and dehydrated once again in acetonitrile. The gel plugs were then dried in a speed vac. Five µl of serum was adsorbed into the dehydrated gel plugs, denatured with DTT at 100 degrees Celsius for five minutes, allowed to cool and alkylated in the dark for thirty minutes with iodoacetamide. The gel plugs were fixed in a solution of 30% ethanol 7% acetic acid for one hour. The gel plugs were washed in acetonitrile, followed by subsequent steps of 20mM ammonium bicarbonate and and acetonitrile before being dried in a speed-vac. PNGase F was diluted with 20mM ammonium bicarbonate (pH 7.0) 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 degrees Celsius. The glycans were eluted from the gel plug by sonication in Milli-Q water three times and the elutant pooled, dried down and labeled with a 2AB dye (Ludger, Oxford, UK) according to the manufacture's instructions. The glycans were cleaned up using paper chromatography and filtered using a 0.22 micron syringe filter. Fluorescently labeled glycans were subsequently analyzed by HPLC using a normal phase column (TSK amide 80 column). The mobile phase consisted of Solvent A (50mM ammonium formate, pH 4.4) and Solvent B (acetonitrile) and the gradient used was as follows: linear gradient from 20%-58% Solvent A at 0.4 ml/minute for 152 minutes followed by a linear gradient from 58%-100% Solvent A for the next 3 minutes. The flow rate was increased to 1.0 ml/minute and the column washed in 100% Solvent A for 5 minutes. Following the wash step the column was equilibrated in 20% Solvent A for 22 minutes 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 the calculation of GU values as described previously, through comparison to known standards and sequential exoglycosidase digestion[17].

2.3 Lectin Fluorophore-Linked Immunosorbent Assay (Lectin FLISA) and GP73 Enzyme-linked immunosorbent assay (ELISA)

Briefly, to remove the fucosylation of the capture antibody (Mouse anti-human AAT or rabbit anti-fetuin (AbD Serotec, Raleigh NC, USA) the antibody was incubated with 10mM sodium periodate for 1 hour at 4 degrees Celsius. An equal volume of ethylene glycol was added and the oxidized antibody brought to a concentration of 10µg/mL with sodium carbonate buffer, pH 9.5. Antibody (5 µg/well) was added to the plate and, following incubation, was washed with 0.1% Tween 20/PBS pH 7.4 and was blocked overnight with 3% BSA/PBS. For analysis, 5 µl of serum was diluted in 95 µl of Heterophilic Blocking Tubes™ (Scantibodies Laboratory, Inc. Santee, CA, USA) and was incubated at room temperature for 1 hour. Subsequently, samples were added to the plates for 2 hours and washed 5 times in lectin incubation buffer (10mM Tris pH 8.0, 0.15M NaCl, 0.1% Tween 20) before fucosylated protein was detected with a biotin conjugated Aleuria aurantia (AAL) lectin (Vector Laboratories, Burlingame, CA). Bound lectin was detected using IRDye™ 800 Conjugated streptavidin and signal intensity 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 Chemicals). It is noted that the lectin-FLISA detects the amount of fucosylation present on an equal amount of captured molecules from each patient sample and is performed in a manner that is independent of the total amount of protein in any given patient. The sandwich ELISA for GP73 was done as previously reported[18].

2.4 Lectin-Western

Briefly, serum was depleted of IgG using protein A/G coated agarose beads and A1AT immunoprecipitated using magnetic Dynabeads coated with monoclonal anti-A1AT (AbD Serotec). Subsequently, A1AT was eluted and resolved via SDS-PAGE. The fucosylated A1AT was detected using biotin conjugated Aleuria aurantia lectin (AAL). Bound AAL was visualized using IRDye™ 800 Conjugated streptavidin and signal intensity measured using the Odyssey® Infrared Imaging System (LI-COR Biotechnology, Lincoln, Nebraska). Subsequently, A1AT was detected using a polyclonal anti-A1AT (Sigma Chemicals) and bound antibody detected using an IRDye™ 700 Conjugated anti-rabbit antibody.

2.5 Statistical analysis

Using a minimum of 19 samples per group, we would have 90% power to detect a 40% change in patient groups with a 33% standard deviation. Therefore 20 samples per group were utilized for this study. Descriptive statistics for patient groups were compared by scatter plots that included the outliers. All values were reported as mean values $\pm$ standard deviation unless otherwise stated. As the data did not follow typical Gaussian distributions, a non parametrical test (two-tailed, 95% confidence, Mann-Whitney Test) was used to determine statistical difference between groups. To evaluate the performance of combining multiple markers, values of multiple markers were inputted into a multiple logistic regression model, in each case the output (predicted value) was between 0 and 1, with 0 being cirrhosis and 1 being cancer. A p value of 0.5 was used as a fixed cutoff and patients were classified as being HCC positive when $p \geq 0.5$, otherwise they were classified as

cirrhotic ($p < 0.5$). 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 analyses were performed using the GraphPad Prism Statistical Software Package (San Diego, CA, USA).

3 Results

3.1 Total serum glycan analysis

Comparative N-linked glycan analysis was performed on individual serum samples from 20 healthy subjects, 20 patients with liver cirrhosis, and 20 patients with liver cirrhosis and HCC, to compare glycan sequencing of total serum to the lectin-FLISA of specific proteins for the detection of HCC. N-linked glycan attached to serum glycoproteins was removed using PNGase F, the glycans labeled with a fluorescent dye and analyzed by normal phase HPLC[11, 13, 15, 19-24]. In this method, each peak on the HPLC represents either individual glycans or mixtures of glycans[25]. Peaks that vary between patient groups can be collected and subsequently analyzed by sequential exoglycosidase digestion to identify peaks that are altered in the specific patient groups. We refer to this approach as “total” serum glycan analysis, because all (total) N-glycans derived from the serum are analyzed, without any depletions or enrichments. Figure 1 shows an example of a normal phase HPLC profile from a representative healthy control subject (top), cirrhotic (middle) and HCC profile (bottom). The asterisks indicate peaks that are altered in the cirrhotic and HCC patient groups. As this figure shows, for these three individuals, peaks 1-10 were elevated in patients with cirrhosis, as well as patients with cirrhosis and HCC. These peaks are consistent with the alterations in IgG that we, and others, have previously reported[15, 26, 27].

The glycans associated with the HCC samples were digested sequentially with neuraminidase (*Arthrobacter ureafaciens*), Almond Meal α -(1-3,4) fucosidase, bovine kidney α -(1,6) fucosidase and Jack Bean Beta-(1-4,6) galactosidase. This sequential exoglycosidase digestion confirmed that peak 27, which was altered in the HCC group, consisted of a tri- sialylated α -1,3 linked fucosylated tri-antennary glycan (see figure 2A-E).

While peak 27 was the major glycan altered in the serum of patients with HCC, there were other large sialylated glycans that correspond to peaks 28-31 that were also altered in HCC. As figure 2B shows, these peaks, when combined with peak 27, comprised a mean of 6.83% (± 2.05) of the total glycan profile in the control patients, 7.96% (± 1.93) of the total glycan profile in the cirrhotic patients and 11.35 % (± 3.12) of the total glycan profile in the HCC patients. There was a statistical difference between the HCC and cirrhosis groups ($p = 0.0008$) and between the HCC and control group ($p = 0.0001$).

Alone, the tri-sialylated α -1,3 linked fucosylated tri-antennary glycan (peak 27) had the ability to differentiate HCC from cirrhosis with an AUROC of 0.765 with a sensitivity of 60% at a specificity of 88%. However, when the level of this peak was simply combined with peaks 28-31 the ability to differentiate HCC from cirrhosis increased to an AUROC of

0.851 with a sensitivity of 73% at a specificity of 88% (Figure 2C). In comparison, AFP had an AUROC of 0.842, with a sensitivity of 55% at specificity of 88%. Using a cutoff of 10% of peaks 27-31 and a cutoff of 20 ng/mL for AFP resulted in a sensitivity of 95% and a specificity of 90%.

3.2 Examination of the desialylated glycan profile.

We also examined the total serum profile following treatment with neuraminidase (de-sialylated profile). As figure 3A shows, when comparing the three patient groups, only one peak was altered in the three patient groups. This peak, peak 19, was 3.01% (± 1.34) of the total glycan profile in the control patients, 4.27% (± 1.53) in the cirrhotic patients and 6.22% (± 1.97) in the patients with HCC (Figure 3B). There was statistical difference between the HCC and cirrhosis group ($p=0.0114$) and between the HCC and control group ($p<0.0001$) but not between any other groups.

Sequential exoglycosidase digestion identified this peak as an α -1,3 fucosylated tri-antennary glycan (data not shown), which is consistent with what others have found in the de-sialylated total serum profile in HCC[28]. This is also the de-sialylated daughter peak of peak 27 in figure 1. The increased level of this glycan had the ability to differentiate HCC from cirrhosis with an AUROC of 0.765 with a sensitivity of 40% at a specificity of 88% (Figure 3C).

3.3 Examination of specific proteins by lectin FLISA or total ELISA

We have previously identified, and examined, a number of proteins that are altered in their amount and glycosylation as a function of HCC[11, 13]. In an effort to compare the results obtained with the total glycan sequencing to the analysis of specific proteins we examined the level of fucosylated fetuin-A (fc-fet), fucosylated alpha-1-anti-trypsin (fc-A1AT), and Golgi-protein 73 (GP73) in this same sample set[12-14, 24, 29]. The results are shown in figure 4. Fc-fet and fc-A1AT were analyzed by lectin-FLISA and GP73 was measured by sandwich ELISA. As figure 4 shows, none of the specific proteins analyzed by either lectin-FLISA or sandwich ELISA had statistically significant increases in the HCC patients, as compared to those with cirrhosis. The best results were obtained with fc-fetuin-A, which had an AUROC of 0.670 at differentiating HCC from cirrhosis with 25% sensitivity at 90% specificity (Figure 4B&D). GP73, which has previously been shown to be a good marker for HCC, was also tested in this sub-set (Figure 4C&D). The mean GP73 level in the control group was 84.2 ng/mL (± 55.91), 295.4 ng/mL (± 165.9) in the cirrhotic group and 280.4 ng/mL (± 108.2) in the HCC group. While there was statistical difference between the control and cirrhotic group ($p<0.0001$) and between the control and HCC group ($p<0.0001$), there was no difference between the HCC and cirrhosis group ($p=0.903$). GP73 had an AUROC of 0.513 at differentiating HCC from cirrhosis with 10% sensitivity at 90% specificity. A combination of these markers did not alter the performance at the detection of HCC (Data not shown).

3.4 Examination of A1AT by Lectin-Western

In an effort to determine if the results with the lectin-FLISA were correct, we analyzed the samples using a modified lectin-immunoblot procedure. In this method, specific proteins are

immunoprecipitated, resolved via one dimension SDS –PAGE and fucosylated forms detected using the Aleuria Aurantia lectin (AAL). The detection is done using a Li-Core Odyssey near infrared imaging system that allows for the detection of AAL binding in one color and the secondary detection of the protein in a second color. An example of this is shown in figure 5. In this figure, A1AT has been immunoprecipitated from the serum of either cirrhotic patients or patients with HCC and the lectin reactive form detected using the AAL lectin (top panel shown in green). The resolved A1AT detected using a polyclonal anti-A1AT and shown in red (middle panel). The combined image is shown in the bottom panel. The relative signal intensity of the AAL (normalized to the level of the A1AT red band) is shown for the cirrhotic and HCC patients in figure 5B. As this figure shows, there is a statistically significant difference in the level of the AAL reactive A1AT in the HCC groups as compared to the cirrhotic group ($p=0.002$). In this gel based assay, fc-A1AT had an AUROC of 0.784 with a sensitivity of 55% at 90% specificity. The performance was not improved when used in combination with AFP (data not shown). The examination of another protein, anti-alpha-1-anti-chymotrypsin (ACT), did not lead to any better performance (AUROC=0.716) than that observed with A1AT.

4 Discussion

Total serum based glycan analysis has been proposed by several groups as a method to detect liver cancer[30]. Most notably, Liu et al. utilized a capillary electrophoresis based technology to examine the de-sialylated profile from patients with HBV associated HCC[28]. In that study, an outer-arm fucosylated tri-antennary glycan was found to be increased in patients with HCC, and this peak along with another, was used to differentiate HCC from cirrhosis. Our work confirms this finding and suggests that looking at other glycans in addition to the sialylated version of this glycan may have added diagnostic value.

It also noted that increases in core fucosylation have also been observed, both in the analysis of specific liver derived glycoproteins and in the total serum of people with HCC[13, 14, 31-39]. The most notable of these is the increased core fucosylation of AFP, which is referred to as AFP-L3[40-42]. AFP-L3 has shown increased specificity as compared to AFP in the differentiation of HCC from cirrhosis[43]. The limitation with AFP-L3 is the limited expression of AFP, which is expressed in roughly half of HCC cases, thus reducing the sensitivity of this assay to less than 50%[43].

In this study, we utilized a simple HPLC based glycan sequencing approach to examine the total serum glycans from controls, patients with cirrhosis and patients with HCC in the background of cirrhosis[17, 20, 23, 25, 44, 45]. We have previously utilized this method to examine de-sialylated glycans associated with serum depleted of either immunoglobulin or the major acute phase proteins[11, 13, 24]. In the current study, we examined total serum without any prior depletion of samples and focused on the sialylated glycan profile. As expected, several major changes were observed in the serum of patients with cirrhosis or HCC in the background of cirrhosis as compared to the controls[16, 46-48]. These changes have been reported by us and by others previously and are most likely associated with alterations in immunoglobulin levels[26, 27]. It is noted that the diagnosis of cirrhosis was

easily distinguishable from the control sample with an AUROC of 0.971 with a sensitivity of 88% and a specificity of 100%.

In contrast, the ability to diagnose HCC in the background of cirrhosis was more challenging. The optimal performance utilized a combination of multiple peaks that consisted of larger branched and sialylated glycans (peaks 27-31, see figures 1-2). These peaks contain mostly tri- and tetra-antennary, highly sialylated glycans, with some having α -1,3- linked (outer arm) fucosylated structures. It is interesting to note that these are the same branched glycans that were previously observed in HCC tissue, suggesting that the cancer itself may be the source of these glycans[49]. The simple summation of these peaks leads to an AUROC of 0.851 at the differentiation of the HCC and cirrhotic groups. More importantly, using a cutoff of 10% for these peaks, the glycan sequencing correctly identified the 4 out of 5 HCC patients with AFP less than 20 ng/mL and 4 out of 4 HCC patients with AFP between 20 and 100 ng/mL of AFP. This is important because at concentrations between 20- 100 ng/mL, AFP generally has poor sensitivity (40-60%) and poor specificity (60-80)%. Thus in many ways, just as important as a biomarker for those that have AFP $\geq$ 20 ng/mL, a biomarker that can clarify and help identify patients with HCC and an AFP concentration in the “grey zone” of 20-100 ng/mL would be of great clinical value.

We also analyzed several other proteins and glycoforms that have shown the ability to distinguish cirrhosis from HCC in other studies[12-14]. Quite surprisingly, while all of the individual protein markers were elevated in the cirrhotic and HCC patient samples, as compared to the controls, none of the markers analyzed had the ability to diagnose HCC with a greater AUROC that was greater than either AFP or the total glycan sequencing. However, for the proteins analyzed by lectin-FLISA, this may be an assay flaw and not necessarily a deficiency of the marker. That is, by lectin-FLISA, fucosylated A1AT had an AUROC of 0.53. However, when this marker was analyzed by a more complicated assay involving the immunoprecipitation of A1AT followed by lectin blotting, the AUROC improved to 0.784, which is similar to what has been observed previously with this marker [12, 14, 37](see figure 5). Unfortunately, we were unable to perform this assay with Fetuin-A or other proteins due to antibody availability but this result suggests that other high throughput assays may need to be developed to allow for analysis of specific protein glycoforms. Indeed, we and others, have noticed greatly increased levels of heterophilic antibodies and bacterial products in patients with liver cirrhosis and these could indeed interfere with the plate based assays[12, 15, 16, 50]. This is currently under investigation.

The observed alteration in glycosylation, increased levels of branched, sialylated and fucosylated (outer-arm) glycans, is similar to what has been observed in other cancers as well and is most likely the result of some cancer induced inflammation[14, 23, 25, 28, 45, 51-55]. One benefit of such a test for the detection of liver cancer is that statistically, those infected with HBV, HCV or have liver cirrhosis, are at the greatest risk for developing liver cancer and not other cancers[2]. Therefore, in this setting, such an assays could have clinical benefit.

In conclusion, the work presented here suggests that the simple analysis of serum linked glycans, without any depletion of serum proteins or exoglycosidase treatment of the released glycan, may have clinical value for the early detection of liver cancer. Although the glycan sequencing detailed here is not suitable as a diagnostic test, new methods have simplified the procedures further and can now allow the entire process to be completed within 4 hours[56]. Thus, it is possible that such test could be used in combination with existing makers in the clinical management of patients at risk for the development of hepatocellular carcinoma.

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Abbreviations

HCC	hepatocellular carcinoma
AFP	Alpha-fetoprotein
GP73	golgi protein 73
Fc-A1AT	fucosylated alpha-1-anti-trypsin
Fc-fet	fucosylated Fetuin-A
AUROC	area under the receiver operator curve

5. References

- [1]. Di Bisceglie AM. Hepatocellular carcinoma: molecular biology of its growth and relationship to hepatitis B virus infection. *Med Clin North Am.* 1989; 73:985–997. [PubMed: 2542706]
- [2]. Block TM, Mehta AS, Fimmel CJ, Jordan R. Molecular viral oncology of hepatocellular carcinoma. *Oncogene.* 2003; 22:5093–5107. [PubMed: 12910247]
- [3]. Marrero JA. Hepatocellular carcinoma. *Curr Opin Gastroenterol.* 2006; 22:248–253. [PubMed: 16550039]
- [4]. Sallie R, Di Bisceglie AM. Viral hepatitis and hepatocellular carcinoma. *Gastroenterol Clin North Am.* 1994; 23:567–579. [PubMed: 7989095]
- [5]. Lok A, McMahon B. Chronic hepatitis B. *Hepatology (Baltimore, Md.)* 2001; 34:1225–1241.
- [6]. Sherman M. Hepatocellular carcinoma: epidemiology, risk factors, and screening. *Semin Liver Dis.* 2005; 25:143–154. [PubMed: 15918143]
- [7]. Anand BS. Cirrhosis of liver. *West J Med.* 1999; 171:110–115. [PubMed: 10510657]
- [8]. Alpert ME, Uriel J, de Nechaud B. alpha fetoglobulin in the diagnosis of human hepatoma. *N Engl J Med.* 1968; 278:984–986. [PubMed: 4171303]
- [9]. Ruoslahti E, Salaspuro M, Pihko H, Andersson L, Seppala M. Serum alpha-fetoprotein: diagnostic significance in liver disease. *Br Med J.* 1974; 2:527–529. [PubMed: 4407283]
- [10]. Di Bisceglie AM, H. JH. Elevations in serum alpha-fetoprotein levels in patients with chronic hepatitis B. *Cancer.* 1989; 64:2117–2120. [PubMed: 2478280]
- [11]. Comunale MA, Lowman M, Long RE, Krakover J, Philip R, Seeholzer S, Evans AA, Hann HWL, Block TM, Mehta AS. Proteomic analysis of serum associated fucosylated glycoproteins in the development of primary hepatocellular carcinoma. *Journal of Proteome Research.* 2006; 6:308–315. [PubMed: 16457596]

- [12]. Wang M, Long RE, Comunale MA, Junaidi O, et al. Novel fucosylated biomarkers for the early detection of hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev.* 2009; 18:1914–1921. [PubMed: 19454616]
- [13]. Comunale MA, Wang M, Hafner J, Krakover J, et al. Identification and development of fucosylated glycoproteins as biomarkers of primary hepatocellular carcinoma. *J Proteome Res.* 2009; 8:595–602. [PubMed: 19099421]
- [14]. Comunale MA, Rodemich-Betesh L, Hafner J, Wang M, et al. 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. [PubMed: 20811639]
- [15]. Mehta AS, Long RE, Comunale MA, Wang M, et al. Increased levels of galactose-deficient anti-Gal immunoglobulin G in the sera of hepatitis C virus-infected individuals with fibrosis and cirrhosis. *Journal of virology.* 2008; 82:1259–1270. [PubMed: 18045939]
- [16]. Balagopal A, Philp FH, Astemborski J, Block TM, et al. Human immunodeficiency virus-related microbial translocation and progression of hepatitis C. *Gastroenterology.* 2008; 135:226–233. [PubMed: 18457674]
- [17]. Guile GR, Rudd PM, Wing DR, Prime SB, Dwek RA. A rapid high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analyzing oligosaccharide profiles. *Anal Biochem.* 1996; 240:210–226. [PubMed: 8811911]
- [18]. Morota K, Nakagawa M, Sekiya R, Hemken PM, et al. A comparative evaluation of Golgi protein-73, fucosylated hemopexin, alpha-fetoprotein, and PIVKA-II in the serum of patients with chronic hepatitis, cirrhosis, and hepatocellular carcinoma. *Clin Chem Lab Med.* 2011
- [19]. Guile GR, Rudd PM, Wing DR, Prime SB, Dwek RA. A rapid high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analyzing oligosaccharide profiles. *Anal Biochem.* 1996; 240:210–226. [PubMed: 8811911]
- [20]. Rudd PM, Dwek RA. Rapid, sensitive sequencing of oligosaccharides from glycoproteins. *Curr Opin Biotechnol.* 1997; 8:488–497. [PubMed: 9265730]
- [21]. Rudd PM, Guile GR, Kuster B, Harvey DJ, et al. Oligosaccharide sequencing technology. *Nature.* 1997; 388:205–207. [PubMed: 9217165]
- [22]. Rudd PM CC, Royle L, Murphy N, Hart E, Merry AH, Hebestreit HF, Dwek RA. A high-performance liquid chromatography based strategy for rapid, sensitive sequencing of N-linked oligosaccharide modifications to proteins in sodium dodecyl sulphate polyacrylamide electrophoresis gel bands. *Proteomics.* 2001; 1:285–289. [PubMed: 11680875]
- [23]. Saldova R, Royle L, Radcliffe CM, Abd Hamid UM, et al. Ovarian cancer is associated with changes in glycosylation in both acute-phase proteins and IgG. *Glycobiology.* 2007; 17:1344–1356. [PubMed: 17884841]
- [24]. Block TM, Comunale MA, Lowman M, Steel LF, et al. Use of targeted glycoproteomics to identify serum glycoproteins that correlate with liver cancer in woodchucks and humans. *Proc Natl Acad Sci U S A.* 2005; 102:779–784. [PubMed: 15642945]
- [25]. Arnold JN, Saldova R, Galligan MC, Murphy TB, et al. Novel glycan biomarkers for the detection of lung cancer. *Journal of Proteome Research.* 2011; 10:1755–1764. [PubMed: 21214223]
- [26]. Watt K, Uhanova J, Gong Y, Kaita K, et al. Serum immunoglobulins predict the extent of hepatic fibrosis in patients with chronic hepatitis C virus infection. *Journal of viral hepatitis.* 2004; 11:251–256. [PubMed: 15117327]
- [27]. Klein A, Carre Y, Louvet A, Michalski JC, Morelle W. Immunoglobulins are the major glycoproteins involved in the modifications of total serum N-glycome in cirrhotic patients. *Proteomics. Clinical applications.* 2010; 4:379–393. [PubMed: 21137058]
- [28]. Liu XE, Desmyter L, Gao CF, Laroy W, et al. N-glycomic changes in hepatocellular carcinoma patients with liver cirrhosis induced by hepatitis B virus. *Hepatology (Baltimore, Md.)* 2007; 46:1426–1435.
- [29]. Marrero JA, Romano PR, Nikolaeva O, Steel L, et al. GP73, a resident Golgi glycoprotein, is a novel serum marker for hepatocellular carcinoma. *Journal of hepatology.* 2005; 43:1007–1012. [PubMed: 16137783]

- [30]. Tang Z, Varghese RS, Bekesova S, Loffredo CA, et al. Identification of N-glycan serum markers associated with hepatocellular carcinoma from mass spectrometry data. *Journal of Proteome Research*. 2010; 9:104–112. [PubMed: 19764807]
- [31]. Buamah PK, Cornell C, Cassells-Smith AJ, Harris AL. Fucosylation of alpha-fetoprotein in hepatocellular carcinomas. *Lancet*. 1986; 1:922–923. [PubMed: 2421123]
- [32]. Aoyagi Y, Suzuki Y, Isemura M, Nomoto M, et al. The fucosylation index of alpha-fetoprotein and its usefulness in the early diagnosis of hepatocellular carcinoma. *Cancer*. 1988; 61:769–774. [PubMed: 2448024]
- [33]. Turner GA. N-glycosylation of serum proteins in disease and its investigation using lectins. *Clinica chimica acta; international journal of clinical chemistry*. 1992; 208:149–171.
- [34]. Aoyagi Y, Suzuki Y, Igarashi K, Saitoh A, et al. Carbohydrate structures of human alpha-fetoprotein of patients with hepatocellular carcinoma: presence of fucosylated and non-fucosylated triantennary glycans. *British journal of cancer*. 1993; 67:486–492. [PubMed: 7679920]
- [35]. Saitoh A, Aoyagi Y, Asakura H. Structural analysis on the sugar chains of human alpha 1-antitrypsin: presence of fucosylated biantennary glycan in hepatocellular carcinoma. *Arch Biochem Biophys*. 1993; 303:281–287. [PubMed: 8390218]
- [36]. Turner GA. Haptoglobin. A potential reporter molecule for glycosylation changes in disease. *Advances in experimental medicine and biology*. 1995; 376:231–238. [PubMed: 8597253]
- [37]. Naitoh A, Aoyagi Y, Asakura H. Highly enhanced fucosylation of serum glycoproteins in patients with hepatocellular carcinoma. *Journal of gastroenterology and hepatology*. 1999; 14:436–445. [PubMed: 10355508]
- [38]. Comunale MA, Lowman M, Long RE, Krakover J, et al. Proteomic analysis of serum associated fucosylated glycoproteins in the development of primary hepatocellular carcinoma. *Journal of Proteome Research*. 2006; 5:308–315. [PubMed: 16457596]
- [39]. Wang M, Long RE, Comunale MA, Junaidi O, et al. Novel fucosylated biomarkers for the early detection of hepatocellular carcinoma. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 2009; 18:1914–1921.
- [40]. Koda M, Hori T, Maeda N, Kato S, et al. Lectin-reactive patterns of markedly elevated serum alpha-fetoprotein in patients with chronic active hepatitis. *Am J Gastroenterol*. 1991; 86:861–865. [PubMed: 1711775]
- [41]. Hirai H, Taketa K. Lectin affinity electrophoresis of alpha-fetoprotein. Increased specificity and sensitivity as a marker of hepatocellular carcinoma. *J Chromatogr*. 1992; 604:91–94. [PubMed: 1379253]
- [42]. Yamashiki N, Seki T, Wakabayashi M, Nakagawa T, et al. Usefulness of Lens culinaris agglutinin A-reactive fraction of alpha-fetoprotein (AFP-L3) as a marker of distant metastasis from hepatocellular carcinoma. *Oncol Rep*. 1999; 6:1229–1232. [PubMed: 10523686]
- [43]. Marrero JA, Feng Z, Wang Y, Nguyen MH, et al. Alpha-fetoprotein, des-gamma carboxyprothrombin, and lectin-bound alpha-fetoprotein in early hepatocellular carcinoma. *Gastroenterology*. 2009; 137:110–118. [PubMed: 19362088]
- [44]. Lauc G, Essafi A, Huffman JE, Hayward C, et al. Genomics meets glycomics-the first GWAS study of human N-Glycome identifies HNF1alpha as a master regulator of plasma protein fucosylation. *PLoS genetics*. 2010; 6:e1001256. [PubMed: 21203500]
- [45]. Saldova R, Fan Y, Fitzpatrick JM, Watson RW, Rudd PM. Core fucosylation and alpha2-3 sialylation in serum N-glycome is significantly increased in prostate cancer comparing to benign prostate hyperplasia. *Glycobiology*. 2011; 21:195–205. [PubMed: 20861084]
- [46]. Callewaert N, Van Vlierberghe H, Van Hecke A, Laroy W, et al. Noninvasive diagnosis of liver cirrhosis using DNA sequencer-based total serum protein glycomics. *Nature medicine*. 2004; 10:429–434.
- [47]. Blomme B, Van Steenkiste C, Callewaert N, Van Vlierberghe H. Alteration of protein glycosylation in liver diseases. *Journal of hepatology*. 2009; 50:592–603. [PubMed: 19157620]

- [48]. Vanderschaeghe D, Laroy W, Sablon E, Halfon P, et al. GlycoFibroTest is a highly performant liver fibrosis biomarker derived from DNA sequencer-based serum protein glycomics. *Molecular & cellular proteomics : MCP*. 2009; 8:986–994. [PubMed: 19181623]
- [49]. Mehta A, Norton P, Liang H, Comunale MA, et al. Increased Levels of Tetra antennary N-Linked Glycan but Not Core Fucosylation Are Associated with Hepatocellular Carcinoma Tissue. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 2012
- [50]. Sandler NG, Koh C, Roque A, Eccleston JL, et al. Host response to translocated microbial products predicts outcomes of patients with HBV or HCV infection. *Gastroenterology*. 2011; 141:1220–1230. 1230, e1221–1223. [PubMed: 21726511]
- [51]. Arnold JN, Saldova R, Hamid UM, Rudd PM. Evaluation of the serum N-linked glycome for the diagnosis of cancer and chronic inflammation. *Proteomics*. 2008; 8:3284–3293. [PubMed: 18646009]
- [52]. Sarrats A, Saldova R, Pla E, Fort E, et al. Glycosylation of liver acute-phase proteins in pancreatic cancer and chronic pancreatitis. *Proteomics. Clinical applications*. 2010; 4:432–448. [PubMed: 21137062]
- [53]. Phillips ML, Nudelman E, Gaeta FC, Perez M, et al. ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-Lex. *Science*. 1990; 250:1130–1132. [PubMed: 1701274]
- [54]. Okada Y, Usumoto R, Muguruma M, Shimoe T, et al. The hepatocellular expression of a carbohydrate antigen ‘sialyl Lewis X’ in chronic hepatitis. A novel histological marker for active hepatic necroinflammation. *Journal of hepatology*. 1990; 10:1–7. [PubMed: 1968474]
- [55]. De Graaf TW, Van der Stelt ME, Anbergen MG, van Dijk W. Inflammation-induced expression of sialyl Lewis X-containing glycan structures on alpha 1-acid glycoprotein (orosomucoid) in human sera. *The Journal of experimental medicine*. 1993; 177:657–666. [PubMed: 7679706]
- [56]. Bones J, Mittermayr S, O'Donoghue N, Guttman A, Rudd PM. Ultra performance liquid chromatographic profiling of serum N-glycans for fast and efficient identification of cancer associated alterations in glycosylation. *Analytical Chemistry*. 2010; 82:10208–10215. [PubMed: 21073175]

Clinical Relevance

Liver disease, in the form of liver cirrhosis and hepatocellular carcinoma (HCC) accounts for 5% of all deaths worldwide. A major reason for this is late (or no) diagnosis of the underlying disease. The currently used marker, serum alpha-fetoprotein (AFP) is elevated in only 40-60% of those with HCC and other markers that can either compliment or replace AFP are highly desired. In the work describe here, we show that large, branched and sialylated N-linked glycans associated with total serum can be used to diagnose cancer independently or in combination with AFP. Surprisingly, these glycans had a greater ability to differentiate HCC from cirrhosis than other assays that examined the glycosylation of specific proteins. Although larger, more diverse patient cohorts will need to be examined, this work strongly suggests that N-linked glycan analysis of the sialylated glycans associated with total serum may have value in the management of those at risk for the development of HCC.

Figure 1. N-linked glycan analysis by normal phase HPLC from a representative control, cirrhotic and HCC patient

A) Normal phase HPLC profile from a representative control patient B) Normal phase HPLC profile from a representative cirrhotic patient. C) Normal phase HPLC profile from a representative HCC patient. The 31 most abundant peaks are labeled. Peaks that were shown to be statistically different in any of the three sample groups are indicated by the asterisks. Peaks 1-10 were found to be increased in the cirrhosis and HCC patient groups. Peaks 27-31 were shown to be altered in the HCC patient groups.

Figure 2. Increased levels of an α -1,3 fucosylated tri-sialylated tri-antennary glycan are associated with HCC serum

(A) The HCC sample shown in figure 1 was sequentially digested with (B) Sialidase (*Arthrobacter ureafaciens*), (C) Almond Meal α -(1-3,4) fucosidase, (D) bovine kidney (α -1,6) fucosidase and (E) Jack Bean Beta-(1-4,6) galactosidase. This sequential exoglycosidase digestion results in the removal of the sialic acid, outer-arm fucosylation, core fucosylation and galactose residues leading to the creation of an the degalactosylated bi-antennary glycan and a degalactosylated tri-antennary glycan as indicated by the arrow. (F) The level of the peak 27-31 glycans in the three patient groups. (G) Receiver operator characteristic (ROC) curves for Peaks 27-31. The AUROC was 0.851 at differentiating HCC from cirrhosis.

Figure 3. De-sialylated N-linked glycan profile of a representative control, cirrhotic and HCC patient

A) Normal phase de-sialylated HPLC profile from a representative control patient, B) a representative cirrhotic patient and C) a representative HCC patient. The 27 most abundant peaks are labeled. Peak 19 (marked with the asterisks,) which was shown to be altered in the HCC group, as compared to the cirrhotic group, was shown by sequential exoglycosidase digestion to be an α -1,3 linked fucosylated tri-antennary glycan. D) Scatter plot indicating the level of this peak in the control, cirrhotic and HCC patient groups. E) Receiver operator characteristic (ROC) curves for this peak. The AUROC was 0.765 at differentiating HCC from cirrhosis.

Figure 4. Scatter plots of the relative levels of (A) fucosylated AAT, (B) fucosylated Fetuin A, or (C) total GP73

(D) Receiver operator characteristic (ROC) curves for each marker individually for the differentiation of cirrhosis from HCC. AFP, as measured in this sample set is included for comparison. In all panels, the line indicates the mean. Analysis of Fc-AAT and Fc-fetuin A were performed via the lectin-FLISA. Total GP73 was analyzed via immunoblotting. AFP was measured using a commercially available AFP ELISA kit.

Figure 5. Lectin-Western analysis of Fucosylated A1AT

A) The levels of fucosylated A1AT were measured by immunoprecipitation of protein followed by immunoblotting with AAL and anti-A1AT. Top panel is the AAL reactive A1AT from 4 representative HCC, 4 cirrhotic and one control sample. In the middle panel, the same blot was probed for A1AT using a poly-clonal anti-A1AT antibody and as this figure shows, all samples have an equal level of A1AT. The bottom panel shows a overlay of the AAL rx bands and the A1AT bands showing the specificity of the assay. B) Scatter plots of the relative levels of fucosylated AAT in the cirrhotic and HCC group. C) Receiver operator characteristic (ROC) curves for fc-A1AT as measured by the lectin-Western blot assay. By this assay, fc-A1AT had an AUROC of 0.784 at differentiating HCC from cirrhosis.

Table I

Sample population characteristics

Disease Diagnosis	Control ¹	Cirrhosis ¹	HCC ¹	p Value
Number	20	20	20	-
Gender (M:F)	10:10	16:04	16:04	-
Etiology (HCV/Alcohol/Crypto) ²	-	9:02:09	9:02:09	-
Age (mean)	53	60.8 (±8.8)	63.7 (±10.4)	0.336
ALT (mean) ³	-	74 (±67.9)	70 (±53.7)	0.551
AST (mean) ⁴	-	89 (±83.1)	111 (±116.5)	0.87
ALK (mean) ⁵	-	139 (±53.2)	215 (±253.9)	0.555
Albumin (mean)	-	3.2 (±0.52)	3.7 (±0.45)	0.0067
Total Bilirubin (mean)	-	1.685 (±1.02)	1.32 (±0.85)	0.2551
MELD (mean) ⁶	-	11.06 (±3.24)	10.5 (±6.04)	0.2784
Childs Class (A/B/C/NA) ⁷		1:6:11:2	12:6:0:2	
HCC Stage (mean) ⁸	-		2.3 (±0.95)	-

¹ Samples were provided from the University of Michigan. See text for more details. Disease diagnosis was determined by MRI or by liver biopsy.

² For Etiology: HCV, hepatitis C virus; alcohol, alcohol induced liver disease; crypto, cryptogenic liver disease.

³ ALT=alanine aminotransferase;

⁴ AST=aspartate aminotransferase;

⁵ ALK=alkaline phosphatase.

⁶ MELD: Model for end stage liver disease. N/A, not available.

⁷ The percent of patients with each Child-Pugh score is given as a percentage in each group.

⁸ 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. Patient's characteristics were analyzed through the use of Chi-Square test, Fisher's exact test or Welch's approximate t test as appropriate. All test were two-sided, and p<0.05 was considered significant.