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Development and application of a novel recombinant *Aleuria aurantia* lectin with enhanced core fucose binding for identification of glycoprotein biomarker of hepatocellular carcinoma

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Abstract

The *Aleuria aurantia* lectin (AAL) derived from orange peel fungus contains five fucose-binding sites that recognizes fucose bound in α -1,2, α -1,3, α -1,4 and α -1,6 linkages to N-acetylglucosamine (GlcNAc) and galactose. Recently, we have created several recombinant AAL (rAAL) proteins that had altered binding affinity to fucose linkages. In this report we further characterize the binding specificity of one of the mutated lectins, N224Q lectin. This lectin was characterized by lectin western blotting and by glycan microarray and shown to have greatly increased binding fucosylated glycan. Subsequently, we used this lectin to identify secreted fucosylated glycoproteins from a fetal hepatic cell line. Proteomic analysis revealed several glycoproteins secreted by the fetal cell line that were bound by N224Q lectin. These findings were confirmed by subsequent proteomic analysis of human serum from control patients or patients with hepatocellular carcinoma. These represent candidate oncofetal markers for liver cancer.

Keywords

Aleuria aurantia lectin; biomarker; glycosylation; hepatocellular carcinoma; liver cancer

1. Introduction

Asparagine (N)-linked glycosylation of secreted and membrane proteins is a common post-translational modification. Both broad changes in the glycan side chains assembled as well as more specific changes to the glycosylation of individual proteins have been associated

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with diseases such as cancer [1]. For instance, fucosylation of *N*-linked glycans has been associated with several types of cancer [2]. An increase in the addition of core α -1,6-linked fucose and α -1,3 outer-arm fucose is associated with the development of hepatocellular carcinoma (HCC) [3–16]. Several of the proteins containing increased fucose have been identified and characterized as potential biomarkers of HCC. In plate based assays, the *Aleuria aurantia* lectin (AAL) was used to detect fucosylation of antibody captured proteins.

Native AAL has five binding pockets for the sugar, each formed between adjacent domains of a six-bladed β -propeller [17–20]. Different sites bind to multiple fucose linkages, including α -1,2, α -1,3, α -1,4 as well as core α -1,6-fucose [17, 18]. However, it is thought that the multiple fucose binding sites of the lectin display differential specificity with respect to the linkages that can bind. We used structural information to re-engineer the wild type lectin, and in this report, characterized the binding of this lectin to fucosylated glycan [21].

Subsequently, as many cancer biomarkers are fetal proteins in origin, we have used this recombinant lectin as an affinity reagent to perform glycoproteomics in an effort to identify potential glycoproteins with altered fucosylation and confirmed the identification of those proteins via a human proteomic analysis of control or HCC serum following extraction with a known core fucose binding lectin.

2. Materials and methods

2.1. Materials

Aleuria aurantia lectin (AAL) was purchased from Vector Laboratories (Burlingame, CA, USA). PNGase F PRIME was obtained from Bulldog Bio (Portsmouth, NH, USA). ChromPure whole molecule of human IgG was purchased from Jackson ImmunoResearch Labs Inc. (West Grove, PA, USA). BSA-fucose and BSA-galactose were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Human cord serum alpha-fetoprotein (AFP) was purchased from Lee Biosolutions, Inc. (Maryland Heights, MO, USA). Recombinant AAL (rAAL) 10 $\times$ His and N224Q were synthesized in house using protocol described previously [21]. Custom peptide of core fucose-specific *Pholiota squarrossa* lectin (PhoSL) was synthesized by CPC Scientific (Sunnyvale, CA, USA) with N-terminus biotin. All reagents used in glycan array were donated by Consortium for Functional Glycomics (CFG) under Glue Grant R24 GM098791 from NIH. PureProteome™ nickel magnetic bead system was purchased from EMD Millipore (Billerica, MA, USA). The fetal liver hepatocyte cBAL111 cells were a gift from Dr. Ruurdte Hoekstra, Academic Medical Center, Tytgat Institute for Liver and Intestinal Research/Experimental Surgery, Amsterdam, the Netherlands. Normal primary human hepatocytes in suspension were obtained through the Liver Tissue Cell Distribution System (Pittsburgh, PA, USA), funded by NIH contract #: HHSN276201200017C. All cell culture media and supplements were purchased from Corning (Corning, NY, USA) unless otherwise indicated. Exoglycosidase enzymes Glyko® α -(1–2,3,6)-Mannosidase, Glyko® α -(1–3,4)-Fucosidase, Glyko® β -(1–4,6)-Galactosidase and Glyko® Sialidase A™ were purchased from ProZyme (Hayward, CA, USA). HyperSep Hypercarb graphite carbon columns was purchased from Thermo Fisher Scientific (Waltham, MA, USA). TSKgel Amide-80 column from Tosoh Bioscience LLC (King of Prussia, PA, USA). HPLC chromatogram were acquired on Waters Alliance HPLC

system complemented with Waters fluorescence detector and Millenium Chromatography Manager software for quantification (Milford, MA, USA). Water was obtained by Milli-Q water purification system from Millipore (Bedford, MA, USA). All other reagents and chemicals were purchased and used from Sigma (St. Louis, MO, USA) as received without further purification.

2.2. Patient samples

Pooled human serum obtained from our clinical collaborator at the Saint Louis University School of Medicine (St. Louis, MO, USA) was used. These samples were collected via a study protocol approved by the Saint Louis University Institutional Review Board and written informed consent was obtained from each subject. Demographic and clinical information is found in a prior publication [9].

2.3. Recombinant lectin expression and peptide synthesis

We have previously expressed and synthesized recombinant AAL (rAAL) as a 526 bp SacI-XhoI fragment into pUC57 shuttle cloning vector (GenScript Inc., Piscataway, NJ, USA) into the T7 expression vectors pET 29-b (Novagen, Darmstadt, Germany) and pQE-T7 (Qiagen, Valencia, CA, USA) with addition sequence of 10 histidine (10×His) tag at the C-terminal end of the rAAL containing asparagine (N) to glutamine (Q) mutation (N224Q) gene sequence. A complete and detailed methods regarding plasmid construction, N224Q expression and purification are described previously by Romano et al. [21]. Custom peptide of core fucose-specific *Pholiota squarrossa* lectin (PhoSL) was synthesized by CPC Scientific (Sunnyvale, CA, USA) using amino acid sequence was obtained from Kobayashi et al. [22]. Recombinant lectins were conjugated with Biotin using the EZ-Link™ NHS-PEG₁₂-Biotin system (Thermo Fisher Scientific) according to manufacturer's suggestion. Lectins were stored in 4°C until further use.

2.4. SDS-PAGE and lectin blotting

Proteins and recombinant lectins were run on SDS-PAGE according to the method of Laemmli [23], using gradient 4–12% Novex Tris-Glycine gels from Life Technologies (Grand Island, NY, USA). Molecular weight SeeBlue Plus2 Pre-stained protein standard was used (Thermo Fisher Scientific; Waltham, MA, USA). Samples were heated for 5 minutes at 100°C in presence of 2-mercaptoethanol. Following SDS-PAGE, gels were stained with Coomassie Blue G250 staining (BIO-RAD; Hercules, CA, USA) or transferred to PVDF membrane for lectin blot. PVDF membrane was blocked using 1× Carbo-Free Blocking solution (Vector Laboratories) for 1 hour. The blots were probed using 1µg/mL biotin labeled lectin in PBS for 1 hour, followed by three-five minute PBS/0.1% Tween-20 washes. The bound lectin was detected using LI-COR Streptavidin iRDye 800 (Lincoln, NE, USA). Gels and blots were scanned using a LI-COR Odyssey Imager.

2.5. Glycan microarray

Mammalian glycan array (version 5.2) were performed by Consortium for Functional Glycomics (CFG) – Emory University, Atlanta, GA, USA under R24 GM098791 glue-grant from National Institute of General Medical Sciences (NIGMS) Large-Scale Collaborative

Project Award. An array containing 609 biologically important glycans were printed in triplicate on NHS-activated slide. Biotinylated recombinant lectins at 50 µg/mL and 5 µg/mL were immobilized and detected by 5 µg/mL cyanine-5 (Cy-5) streptavidin. For Cy-5 detection, a ProScanarray microarray scanner with excitation at 649 nm and emission 670 nm was used. The scanned image was analyzed with ScanArray Express software. Data were normalized to percentage of the highest RFU to each glycan at certain lectin concentration to obtain average binding. Data presented are average signal intensity and error bar indicates standard deviation. Data collected from this experiment are available open-access for scientific community under CFG request# 3101. Detailed method described by Song et al. [24].

2.6. Cell culture of cBAL111 and primary human hepatocytes

cBAL111 fetal liver hepatocytes were cultured in Dulbecco modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS (Corning), 1 µM Dexamethasone (Sigma), 6.7 ng/mL of a mix containing Insulin, Transferrin, Selenium and 100 U/mL penicillin and 100 µg/mL streptomycin (Corning). Cells were grown at 37°C in the presence of 5% CO₂. These cells were grown to confluence in 225 cm² tissue culture flask (Corning). When confluent, the cells were washed twice with cold PBS and serum-free media, then placed into serum-free media for 24 hours. The secreted proteins were collected and filtered through 0.45 µm syringe filter, and buffer exchanged into PBS using Amicon Ultra-15 centrifugal filter units with 10 kD molecular weight cut-off (Millipore) at 4000 × g at 4°C in swinging bucket rotor of Eppendorf 5804R centrifuge.

2.7. Glycan sequencing

N-glycans from 250 µg of protein was released by overnight incubation of 30 U PNGase F and purified using HyperSep Hypercarb porous graphitic carbon columns and dried down by SpeedVacTM vacuum concentrators (Thermo Fisher Scientific). Cleaned *N*-glycans were labeled fluorescently with 2-aminobenzamide (2-AB) and separated on normal phase Waters Alliance HPLC system as previously described [21–26]. Additionally, samples were subjected to exoglycosidase digestion (mannosidase, sialidase, fucosidase and galactosidase, sequentially) for calculation of glucose unit (GU) value and compared to GlycoBase 3.2.4 database (National Institute for Bioprocessing Research and Training; Dublin, Ireland).

2.8. Lectin enrichment

250 µg recombinant His-tagged AAL N224Q was bound to PureProteomeTM Nickel magnetic beads (EMD Millipore) according to manufactures instructions. Secreted proteins from cBAL111 were collected in a serum free media, concentrated using a 10K buffer exchanged into PBS and 250 µg of protein was incubated with the N224Q-bead slurry overnight at 4°C. Unbound fractions were collected through a series of TBS washes that were collected and pooled. Bound proteins were eluted with 200 mM L- (–) Fucose (Sigma). Unbound and bound fractions were concentrated and buffer exchanged into TBS using 10,000 molecular weight cut off spin filter (EMD Millipore) at 10,000 × g at 4°C. Bound and unbound samples were prepared for mass-spectrometry sequencing.

Biotin labeled PhoSL (250 µg) was bound to 95 µL of streptavidin-agarose beads according to manufactures instructions. Human sera, 20 sample of each cirrhotic and hepatocellular carcinoma (HCC), were pooled, protein A/G depleted of IgG, and incubated with PhoSL-agarose slurry. Unbound proteins were collected through a series of TBS washes, collected and pooled. The bound fraction was eluted using 200 mM Fucose with a 48-hour incubation at 4°C. Unbound and bound fractions were buffer exchanged with PBS as above.

2.9. Mass spectrometry

Protein concentrations from bound and unbound lectin pools were determined through Bradford Assay and 1.5 µg of protein was digested with trypsin as described elsewhere [25, 26]. Trypsin digested peptide were concentrated and desalted using ZipTip C18 (Millipore) according to manufacturer's protocol. Prepared samples were analyzed by the Biological Mass Spectrometry Facility at Rutgers, the State University of New Jersey (Piscataway, NJ, USA) using a Velos LTQ Orbitrap tandem mass spectrometer coupled to a Dionex Ultimate 3000 RLSCnano System (Thermo Scientific). Samples were solubilized in 5% acetonitrile/0.1% TFA, and loaded on to a fused silica trap column of 100 µm × 2 cm packed with Magic C18AQ, 5 µm 200Å (Michrom Bioresources Inc, Auburn, CA, USA). After washing for 5 min at 10 µl/min with solvent A (0.2% formic acid), the flow rate was reduced to 300 nL/min and the trap brought in-line with a homemade analytical column (Magic C18AQ, 3µm 200 Å, 75 µm × 50cm) for LC-MS/MS. Peptides were eluted using a segmented linear gradient from 4 to 90% B (B: 0.08% formic acid, 80% ACN): 4% B for 5min, 4–15% B for 19 min, 15–25% B for 40 min, 25–50% B for 55 min and 50–90% B for 8 min. Mass spectrometry data was acquired using a data-dependent acquisition procedure with a cyclic series of a full scan acquired in Orbitrap with resolution of 60,000 followed by top 20 peaks fragmented and scanned in linear ion trap with a repeat count of two and the dynamic exclusion duration of 60 seconds. The LC-MS/MS data was searched against the most up-to-date human complete protein database (ensembl.org) using a local version of the Global Proteome Machine (GPM cyclone, Beavis Informatics Ltd; Winnipeg, Canada) with carbamidoethyl on cysteine as fixed modification and oxidation of methionine and tryptophan as variable modifications using a 10 ppm precursor ion tolerance and a 0.4 Da fragment ion tolerance.

3. Results

In our previous work, in an effort to create fucose-binding lectins with increased specificity and sensitivity, we constructed recombinant (r)AAL molecules containing specific point mutations and domain changes in an effort to alter binding specificity [21]. Briefly, structural analysis of AAL [18–20] has shown that the 2 and 4 binding sites have glutamine (Q) residues in their fucose binding pockets that coordinate with other amino acids within the pocket to maintain a secondary structure required for high affinity fucose binding. In binding sites 3 and 5, an asparagine (N) is found in this position. As the side chain on N is one carbon shorter than Q, the 3 and 5 binding sites lack critical hydrogen bond contacts required for high affinity fucose binding [19]. Thus, we changed the 224 asparagine in binding site 5 to glutamine to create rAAL N224Q. This mutation was predicted to increase the hydrogen bonding potential of the 5 site for fucose containing targets. This protein was

expressed in *E. coli* as His-tagged protein and purified via a two column FPLC IMAC procedure which yields > 90% pure fucose free recombinant AAL. Figure 1A shows a SDS-PAGE Coomassie stain analysis of wild-type AAL purchased from Vector Laboratories (36 kD), purified wild-type rAAL-10×His, and the rN224Q mutant. The larger size of rAAL-10×His and rN224Q is due to C-terminus 10 histidine tag found on the recombinant lectins (10×His). In addition, the novel *Pholiota squarrosa* lectin (PhoSL) [22], which specifically recognizes α -1–6 fucosylation (core-fucosylation) was synthesized but not examined via SDS-PAGE, as it was too small (4.5 kD) to be observed on this gel (data not shown).

Figures 1B–E shows the use of these lectins as affinity reagents in a lectin blot. Figure 1B shows the target antigens analyzed by lectin-western. Target antigens shown in Panel 1B included human IgG (with only a single α -1,6 linked fucose), bovine serum albumin (BSA) conjugated with α -1,3 linked fucose, BSA conjugated with galactose, and cord blood purified alpha-fetoprotein (AFP), which contains only α -1,6 linked fucose [21, 27]. In all cases 10 μ g of target antigen was resolved via SDS-PAGE and detected using 1 μ g/mL of lectin. As panel 1C shows, wild type AAL binds all fucose linkages regardless of linkage. In contrast, the N224Q mutant had strong binding to the α -1,6 fucosylated IgG and AFP but very weak binding to the α -1,3 linked fucosylated glycoproteins (Fig. 1D). This was similar to the PhoSL lectin, which had binding to the core fucosylated IgG and weaker binding to core fucosylated AFP and no binding to the α -1,3 linked fucosylated glycoproteins [22]. It is noted that limited washing (1×) of blots resulted in binding of the rN224Q lectin to the α -1,3 and α -1,4 linked fucosylated glycoproteins, but this was lost with subsequent repeated washings (3×). This is consistent with the idea that N224Q, like what has been reported for *Aspergillus oryzae* L-fucose-specific lectin (AOL), has irreversible binding to core fucosylated glycan[28].

The N224Q lectin, as it showed promise as core fucose binding lectin, was subsequently examined in the Consortium for Functional Glycomics (CFG) glycan array. The binding of the N224Q lectin or the recombinant wild-type control (concentrations of 50 μ g/mL and 50 μ g/mL) was tested on Version 5.2 of the Array (Fig. 2). Figure 2 shows a comparison of the binding of 5 μ g/mL of either the rAAL (Fig. 2A) or rN224Q lectin (Fig. 2B) to the glycans on the array. For both rAAL and the rN224Q, very limited binding to non-fucosylated glycan was observed at any lectin concentration (< 20 RFU). Greatest binding for rAAL and rN224Q was observed for Fuca1–2Gal, Fuca1–3GlcNAc Fuc β 1–3GlcNAc, Fuca1–4GlcNAc and Fuca1–6GlcNAc-linked oligosaccharides. As figure 2 shows, significantly increased binding was observed for a number of glycan and a number of these are highlighted in boxes within Figure 2B and the specific glycans are shown in Figures 2C–E. Greatly increased binding with N224Q was observed with non *N*-linked glycan containing α 1–2,1–3 and 1–4-linked glycans (Fig. 2C–D). In the case of *N*-linked glycans, the greatest increase in the binding of N224Q was observed with *N*-linked corefucosylated glycan (Fig. 2E).

Many, if not most, biomarkers of cancer are fetal expressed genes that are de-repressed, or over-expressed, in cancer cells. AFP for example, has long been associated with hepatocellular carcinoma (HCC). Importantly, this molecule also becomes core fucosylated

in HCC. Thus, we reasoned that other such oncofetal proteins might also become core fucosylated. To test this possibility, the glycosylated proteins produced by a human fetal hepatocyte cell lines were characterized. The cell line cBAL111 was established by using the human telomerase reverse transcriptase to immortalize human fetal hepatocytes [29]. These cells were described as being positive for both albumin and AFP. Cells were cultured as described in the Materials and methods, and then washed and transferred into medium lacking serum or other protein supplements. After 24 hours, supernatants and cell lysates were collected and protein associated *N*-linked glycans were released enzymatically using PNGase F. After labeling the sugars at the reducing end with 2-AB dye individual glycan species were separated by neutral phase HPLC [6, 10, 14]. Glycan profile chromatograms are shown in Figure 3. A standard curve using the homopolymer dextran was used to convert the elution time into glucose units is shown at the bottom of the figure.

The glycan profile reveals the total released *N*-glycans present in the supernatant. Below, a series of sequential exoglycosidase digestions were performed to identify the individual glycans present in the supernatant (Fig. 3A). Treatment with sialidase revealed that multiple species contained terminal sialic acid residues (Fig 3A, second chromatogram from the top). Sialic acid removal simplified the profiles and allows us to conclude that the most abundant species in the cBAL111 supernatant is a triantennary A3G3 (GU 8.46) glycan that contributed 51.5% of the total profile. Treatment with almond meal fucosidase, which removes terminal α -1,2- and α -1,3-linked fucose residues did not change the profile, implying a lack of structures containing these fucosylated glycan. In contrast, treatment with bovine kidney fucosidase removed peaks at GU 7.63, representing a biantennary glycan with a core α -1,6-linked fucose(F (6) A2G2) that contributed 13.8% of the total glycan profile. Two minor species, representing core fucosylated tri- and tetra-antennary structures, were also reduced in height by this enzymatic treatment, although the tetra-antennary structure may have been incompletely digested. In summary, the analyses reveal that slightly more than half of the total glycan secreted by cBAL111 is a triantennary non-fucosylated structure. This is followed by the non-fucosylated and the fucosylated biantennary structures, each representing approximately 13% of the total glycan profile. It is interesting to note that glycan analysis of the cBAL111 cell lysate is very similar (Fig. 3B). However, this is in sharp contrast to what is seen in primary human hepatocytes (Fig. 3C), which primarily secrete proteins with *N*-linked biantennary non-fucosylated structures.

Based on the presence of core fucose present in the cBAL111 supernatants, we reasoned that fucose-binding lectins could be used to capture and identify fucosylated proteins. The rAAL variant N224Q was chosen to use[30] as an affinity reagent for isolation of protein from cBAL111 supernatant; the workflow is diagrammed in Supplementary Figure S1. Both bound and unbound fractions were collected, digested with trypsin and subjected to LC MS/MS analysis as we have done previously [10]. A complete list of all the glycoproteins that were enriched in the N224Q bound fraction versus the unbound fraction are shown in Supplementary Table S1. Thirty of the top identified proteins in the fucosylated secreted proteome of cBAL111 cells are shown in Table 1. In this list, proteins such as Fibronectin (FN1), which has recently been shown to be found in the human serum fucosylated proteome [31] and golgi-protein 73 (GOLM1), which was also originally identified as part of the human serum fucosylated proteome [15], are found highlighting the potential value of

the identified proteins as biomarkers of HCC. The peptide list used to identify proteins is found in Supplementary Table S2 (bound) and S3 (unbound).

As the glycan array indicated that the N224Q lectin can bind non core fucosylated glycan we performed a secondary confirmatory proteomic study, on pooled sera from patients with liver cirrhosis or pooled sera from matched HCC patients (also with cirrhosis) using the core fucose-binding lectin PhoSL and subjected these samples to identical LC MS/MS analysis. As Table 2 shows, 10 of the identified proteins found as part of the fucosylated proteome from the fetal cell line were also found associated with the core fucosylated proteome in human HCC. The peptide list used to identify proteins is found in Supplementary Table S3. These are considered further in the discussion.

4. Discussion

We have described the production of a novel variant of a recombinant AAL lectin with improved specificity for α -1,6-linked fucose relative to the wild type lectin. Specificity was confirmed via lectin blotting (Fig. 1) and binding to glycoprotein and glycan array (Fig. 2). We then used this reagent to affinity capture core fucosylated glycoproteins from the supernatant of a human fetal hepatocyte derived cell line (Fig. 3). AFP, one of the few biomarkers for HCC, is produced by the fetal liver cells [29]. Thus, we reasoned that other co-regulated fucosylated proteins might also prove useful as biomarkers. Proteomic analysis of bound versus unbound proteins revealed enrichment for several known secreted glycoproteins (Table 1) that have been associated with HCC, by us and by others. These include the proteins fibronectin [31], complement C1 R subcomponent, complement C1 S subcomponent [10], angiotensinogen [10], clusterin [20] and GP73[32]. We have previously reported on the association of differentially glycosylated clusterin (CLU, also known as Apo-J) with liver disease [8]. In that study, we did not observe changes in core fucosylation with HCC compared to normal or cirrhosis. Instead, a decrease in the level of β -1,4 triantennary glycan was associated with increasing disease severity. However, it is conceivable that a shift in core fucosylation at a single site is being detected with the PhoSL lectin in the HCC vs. cirrhotic samples.

The most highly represented glycoprotein that fit the criteria of being bound to N224Q and elevated in HCC is fibronectin (FN1). The form produced by hepatocytes is secreted into the circulation and is typically present in plasma at 0.3 mg/ml. Thus, the protein itself is unlikely to be useful as a biomarker, but the core fucosylated form(s) has already shown potential as a biomarker of HCC [31]. Core fucose is much more prevalent on the cellular form of fibronectin versus the plasma form produced by normal adult hepatocytes [33], perhaps reflecting the fetal origin of the cBAL111 cells. A truncated version of FN1, containing only 657 amino acids of the N-terminus of the protein, has been termed migration stimulating factor (MSF), and is considered an oncofetal form [34]. However, inspection of the fibronectin peptides detected revealed that most map more C-terminally, and are likely not derived from MSF. An isoform of FN1 has previously been described as being an "oncofetal" antigen, based on an antibody that recognizes a complex epitope comprising both protein and O-linked oligosaccharide in a region of the protein that undergoes alternative splicing [35].

Like fibronectin (FN1), fibulin (FBLN1), thrombospondin1 (TSBS1), and laminin subunit gamma-1 (LAMC1) are large extracellular matrix glycoproteins. While fibulin has been reported to be core fucosylated [36], only O-linked fucose has been reported for thrombospondin [37], and to our knowledge, there have been no reports of laminin fucosylation. Changes in expression of all three have been associated with HCC [38–41]. A glycoproteomics study of sera from HCC patients vs. controls identified a reduction of thrombospondin1 with HCC [42]. Clusterin was also reduced in HCC in that study, along with C1R, whereas galectin-3 binding protein was increased in HCC along with AFP. We also detected more of the latter protein in HCC vs. cirrhotic (Table 1), but cannot distinguish between changes in protein level vs. changes in amount of core fucose. Galectin-3 binding protein (LGALS3BP) was reported to be core fucosylated in plasma [36], and at least one site of core fucosylation is specifically associated with HCC. Increases in the ligand galectin-3 also have been associated with HCC [43]. Galectin-3 recognizes the oncofetal Thomsen-Friedenreich disaccharide antigen [44].

C1R and C1S are both components of C1, the first step in the classical complement activation pathway and have been previously identified as being part of the fucosylated serum proteome [10]. Similarly, angiotensinogen (AGT), a key constituent of the renin-angiotensin system[45], was previously identified as part of the fucosylated serum proteome in HCC patients[10]. In addition, angiotensinogen has been linked to HCC in a mouse model of HCC [46]. Finally, low-density lipoprotein receptor-related protein (LRP1) is actually a membrane anchored glycoprotein. However, it was detected in plasma [36], suggesting that some is lost from the surface. That study also identified LRP1 as being core fucosylated, consistent with our results. However, the very low representation in the HCC serum makes its use as a biomarker uncertain.

In conclusion, we have performed glycoproteomics analysis of a fetal liver cell line in an effort to identify potential biomarkers of HCC. Conformation of many of the identified proteins was made through the subsequent analysis of human sera from those with HCC. Studies are underway to determine the ability of these proteins to act as biomarkers of HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Purification and characterization of recombinant lectins

(A) SDS-PAGE and Coomassie Blue detection of purchased AAL (Vector Lab) or recombinant AAL (rAAL) with 10×His tag, rAAL with 10×His and N224Q mutation. (B) SDS-PAGE and Coomassie Blue staining of glycoprotein antigens. From left to right, human IgG (IgG), α -1,3 fucose (F-BSA), galactosylated BSA (G-BSA), alpha fetoprotein (AFP). (C) Lectin blotting of samples in Panel B with rAAL. Lectin blotting of samples in Panel B with the N224Q lectin (E) Lectin blotting of samples in Panel B with PhoSL. In all cases lectins were biotinylated and visualized using LI-COR Odyssey Infrared scanner under the same intensity (5.0).

Figure 2. Glycoconjugate microarray performed by the Consortium for Functional Glycomics (CFG)

5 $\mu\text{g/mL}$ of the rAAL (A) or N224Q lectin (B) on the 609 glycan array (V5.2). Areas of greater binding of the N224Q lectin are shown within a box. The net intensity value of each glycoconjugate is indicated. The data presented are average $\pm$ standard deviation of triplicate determination. (C–E). Specific glycan structure that showed increased affinity for N224Q lectin.

Figure 3. N-linked glycans sequencing of secreted proteins from the cBAL111 fetal liver cell line
 (A) Secreted N-glycan from serum-free cBAL111 fetal liver cell were released by PNGase F, followed by sequential exoglycosidase digestion starting with mannosidase, sialidase, galactosidase and fucosidase. (B) PNGase F, mannosidase and sialidase treatment of cBAL111 cell lysate, and largest peak observed was A3G3 triantennary glycans (C) Similarly primary human hepatocyte were treated with PNGase F, mannosidase and sialidase. Largest peak observed in adult liver is (A2G2) biantennary glycans.

Table 1

Top 30 cBAL111 Proteins associated with the N224Q proteome

Gene ¹	Protein name ²	N224Q Bound (Spectral Counts/Unique Peptides) ³	N224Q unbound (Spectral Counts/Unique Peptides) ³	Number of N- linked sites ⁴
FN1	Fibronectin	164 (75)	82 (41)	7
TIMP1	Tissue metalloproteinase inhibitor 1	134 (33)	37 (3)	2
IGFBP4	Insulin-like growth factor-binding protein 4	122 (22)	88 (1)	1
FBLN1	Fibulin	118 (31)	7 (1)	3
FSTL1	Follistatin-related protein 1	100 (22)	19 (5)	3
SERPINE1	Plasminogen activator inhibitor 1	90 (22)	69 (17)	3
C1R	C1 R subcomponent	73 (25)	1 (0)	4
CTSB	Cathepsin B	66 (21)	3 (2)	1
PSAP	Prosaposin	61 (23)	7 (2)	5
CTSD	Cathepsin D	57 (24)	30 (3)	2
NID2	Nidogen-2	57 (30)	22 (8)	5
AHNAK	Neuroblast differentiation-associated protein AHNAK	56 (83)	7 (1)	0
GRN	Granulins	54 (22)	2 (0)	5
CLU	Clusterin	51 (16)	0 (0)	7
THBS1	Thrombospondin-1	49 (24)	13 (3)	4
AGT	Angiotensinogen	47 (15)	15 (8)	4
LGALS1	Galectin-1	44 (9)	20 (4)	0
CLSTN1	Calsyntenin-1	43 (22)	19 (2)	3
LGALS3BP	Galectin-3-binding protein	42 (16)	0 (0)	7
FBN1	Fibrillin-1	41 (17)	0 (0)	15
ADAM9	Disintegrin and metalloproteinase domain-containing protein 9	36 (18)	1 (0)	6
C1S	C1 s subcomponent	34 (15)	6 (0)	2
LRP1	Prolow-density lipoprotein receptor related protein 1	33 (19)	1 (0)	52
STC2	Stanniocalcin-2	32 (11)	3 (1)	1
CD109	CD109 antigen	30 (14)	0 (0)	10
NAGLU	Alpha-N-acetylglucosaminidase	29 (14)	0(0)	6
TINAGL1	Tubulointerstitial nephritis antigen	25 (15)	0 (0)	2
LOXL3	Lysyl oxidase homolog 3	25 (12)	0 (0)	5
BMP1	Bone morphogenetic protein 1	22 (13)	0 (0)	5
GOLM1	Golgi Protein 73	10 (7)	0 (0)	3

¹) Gene name.²) Protein name.³) Spectral counts and number of peptides identified by LC MS/MS.⁴) The number of N-linked sites on the identified protein.

Table 2

Proteins found in both the lectin bound cBAL111 secreted fucosylated proteome and found in the human HCC serum fucosylated proteome.

Gene ¹	Protein name ²	cBAL111 N224Q bound ³	cBAL111 N224Q unbound ³	Cirrhotic serum PhoSL Bound ³	HCC serum PhoSL Bound ³
FN1	Fibronectin	164	82	67	76
FBLN1	Fibulin	118	7	36	52
C1R	C1 r subcomponent	73	1	8	34
CLU	Clusterin	51	0	44	75
THBS1	Thrombospondin 1	49	13	0	11
AGT	Angiotensinogen	47	15	19	29
LGALS3BP	Galectin-3-binding protein	42	0	30	60
C1S	C1 s subcomponent	34	6	24	48
LRP1	Prolow-density lipoprotein receptor related protein 1	33	1	0	1

¹) Gene name.

²) Protein name.

³) Spectral counts for the identified protein by LC MS/MS in the indicated sample.