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N-linked glycan branching and fucosylation are increased directly in HCC tissue as determined through in situ glycan imaging.

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Abstract

Hepatocellular carcinoma (HCC) remains as the fifth most common cancer in the world and accounts for more than 700,000 deaths annually. Changes in serum glycosylation have long been associated with this cancer but the source of that material is unknown and direct glycan analysis of HCC tissues has been limited. Our laboratory previously developed a method of *in situ* tissue based N-linked glycan imaging that bypasses the need for microdissection and solubilization of tissue prior to analysis. We used this methodology in the analysis of 138 HCC tissue samples and compared the N-linked glycans in cancer tissue with either adjacent untransformed or tissue from patients with liver cirrhosis but no cancer. Ten glycans were found significantly elevated in HCC tissues as compared to cirrhotic or adjacent tissue. These glycans fell into two major classes, those with increased levels of fucosylation and those with increased levels of branching with or without any fucose modifications. In addition, increased levels of fucosylated glycoforms were associated with a reduction in survival time. This work supports the hypothesis that the increased levels of fucosylated N-linked glycans in HCC serum are produced directly from the cancer tissue.

Graphic Abstract

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Introduction:

Changes in N-linked glycosylation are known to occur with the development of many cancers, including hepatocellular carcinoma (HCC) [1–13]. In our previous work, we examined serum for protein glycoforms that are altered in liver cancer and have documented significant alterations in serum N-linked glycosylation with the development of HCC [14–23]. Specifically, the alterations are increased levels of alpha-1,3 and alpha-1,6 linked fucosylation found on bi, tri and tetra-antennary glycans and to a lesser extent alterations in high mannose and tetra-antennary glycans [14–23]. Importantly, many of these changes are now being developed as serum-based biomarkers of HCC. However, the origins of these glycans in human HCC are unknown and glycan analysis of tissue is complicated by the mixing of different cell types and the loss of protein during processing. To address these limitations, we have developed a method of tissue-based glycan imaging that allows for both qualitative and quantitative in situ N-linked glycan analysis on tissue using matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) [24–27]. This method bypasses the need for microdissection and solubilization of tissue prior to analysis. When matrix is applied across the tissue section, desorption can be targeted to specific “points” in a pattern and the data rasterized. The resulting spectra can then be used to generate two-dimensional heat maps of hundreds of glycans directly from the surface of a tissue section. These molecular maps display the relative abundance and spatial distribution of these molecules. Thus, MALDI tissue profiling has the power to link the molecular detail of mass spectrometry with molecular histology, generating mass spectra correlated to locations within a thin tissue section. This method is becoming a robust technique for the analysis of glycan *in situ* [24–33]. In this study, we used this methodology in the analysis of two tissue microarrays (TMA). The first TMA consisted of 48 HCC tissue samples, 22 cirrhotic tissue samples and 5 healthy control tissue samples. The second TMA consisted of 90 HCC tissue samples and 90 control adjacent tissue samples. MALDI glycan imaging has identified 10 glycans that were significantly increased in the HCC TMA samples when compared to cirrhotic tissue (TMA #1) or to non-transformed adjacent tissue (TMA#2). These glycans fell into two major classes, those with increased levels of fucosylation and those with increased levels of branching without any fucose modifications. The relevance of this finding to serum based biomarkers and the potential prognostic role of these glycans is discussed.

Materials and Methods

Materials:

Trifluoroacetic acid, Harris-modified hematoxylin, and α -cyano-4-hydroxycinnamic acid (CHCA) were obtained from Sigma Aldrich (St. Louis, MO). HPLC grade methanol, ethanol, acetonitrile, xylene, hydrogen peroxide and water were obtained from Fisher Scientific (Pittsburgh, PA). Tissue Tack microscope slides were purchased from Polysciences Inc (Warrington, PA). Citraconic anhydride and SafeClear II was purchased from Thermo Scientific (Bellefonte, PA). Recombinant Peptide N-Glycosidase F (PNGase F) from *Flavobacterium meningosepticum* was obtained, expressed, and purified as previously described [34], but is also available commercially as PNGase F Prime™ from

Bulldog Bio (Portsmouth, NH). Universal Antigen Retrieval Reagent was purchased from R&D Systems (Minneapolis, MN).

Tissues and Tissue Microarrays:

Normal and hepatocellular carcinoma whole liver tissue samples were purchased from ProSci Inc. (Poway, CA) and cirrhotic whole liver tissue was purchased from BioChain (Newark, CA). All tissue microarray (TMA) slides were purchased from US Biomax (Rockville, MD) as unstained formalin fixed paraffin embedded (FFPE).

The first TMA (Catalog Number: BC03117) contained 80 cores. Forty-eight cases of HCC with a history of Hepatitis B virus (HBV) infection, five cases of cholangiocellular carcinoma with a history of Hepatitis B virus (HBV) infection, 22 cases of liver cirrhosis with a history of Hepatitis B virus (HBV) infection and five normal hepatic tissue cores. These cores were 1.5 mm in diameter and 5 μ m thick. For the purpose of this study, the cholangiocellular carcinoma tissue was included in any analysis.

The second TMA slide (Catalog Number: HLiv-HCC180Sur-04) contained 90 cases of HCC with tumors ranging from stage 1 (early) to 4 (late) and grades G1 (well-differentiated) to G3 (poorly differentiated). All HCC tissues had matched un-transformed adjacent tissue. Along with this, survival data and pathology diagnosis was included for each case. The cores were cut at a 1.5 mm diameter and a thickness of 4 μ m.

Washes for Deparaffinization

As described previously [26], FFPE TMA slides were heated at 60°C for 1 hr and cooled to room temperature prior to deparaffinization. The slides were washed with xylene to remove the paraffin and then rehydrated using a series of water and ethanol washes. Antigen retrieval was performed using citraconic anhydride (Thermo Scientific) as the buffer and placed in a vegetable steamer for 30 minutes. The buffer was then cooled to room temperature and buffer exchange was performed to replace the slides in 100% water. Finally, the slides were desiccated until dry.

Enzymatic Digestion and Matrix Deposition

A M3 TM-Sprayer™ Tissue MALDI Sample Preparation System (HTX Technologies, LLC) was used to spray 0.5 mL of 0.1 μ g/ μ L aqueous solution PNGase F as previously described [26]. Following the spray, the slides were placed in a humidified chamber and incubated at 37°C for 2 hours. Slides were then desiccated and dried prior to matrix application. The matrix was assembled using α -cyano-4-hydroxycinnamic acid (0.042 g CHCA in 6 mL 50% acetonitrile/49.9% water/0.1% TFA) and sprayed using the same M3 TM-Sprayer.

N-Glycan Imaging using MALDI-IMS:

The slides were analyzed for released N-glycan ions using a Solarix dual source 7T FTICR mass spectrometer (Bruker Daltonics, m/z 500–5000) with a SmartBeam II laser operating at 1000 Hz and with a laser spot size of 25 μ m. 200 laser shots were collected for each pixel, with a time domain of 512K. This resulted in a resolving power of 160,000 at m/z 400. A total of 23,145 positions were collected for TMA #1 and 44,533 positions collected for

TMA #2. Afterwards, the data was analyzed using FlexImaging 4.0 (Bruker Daltonics) and SCiLS Lab (Bruker Daltonics, version 2017b) to create images and determine regions of differentially expressed glycans, all normalized to total ion current. A signal to noise (S/N) ratio of 9 was used and peaks were manually picked within FlexImaging 4.0. The resulting glycans were given composition using an in-house database based on collected m/z values and checked against the database from GlycoWorkbench based on m/z and composition [35]. Possible and likely structures for visual representation were built using GlycoWorkbench as well.

Lectin Histochemistry:

The tissue slides were deparaffinized by using PROTOCOL SafeClear II clearing agent, then rehydrated in a series of ethanol washes at 3 minutes per each step (100%, 90%, 70%) and fully hydrated in deionized water for 5 minutes. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide, followed by a 92°C heated antigen retrieval using Universal Antigen Retrieval Reagent (Dako, Carpinteria, CA). The slides were then fixed with 4% formaldehyde solution followed by a permeabilization step using 0.5% IGEPAL CA-630. Following the permeabilization step, for blocking non-specific binding, the slides were blocked once again with serum-free protein block (Dako), supplemented with Streptavidin/Biotin blocking solution to block endogenous biotin, biotin receptors, and streptavidin binding sites in tissues (Vector Laboratory, Burlingame, CA). Streptavidin horseradish peroxidase ready to use solution (Vector Laboratory) was used to detect biotinylated recombinant *Aleuria aurantia* N224Q (rAAL N224Q) lectin bound to the tissue, and visualization was further developed using 3,3'-diaminobenzidine (DAB) Chromogen (Dako). The N224Q lectin is a modified *Aleuria aurantia* lectin with increased binding to core fucosylated glycan (Herrera et al., manuscript submitted). Lectin was applied for 1 hour at room temperature at a concentration of 0.5 $\mu\text{g/mL}$ in background reducing antibody diluent (Dako, Carpeinteria, CA). After incubation, slides were washed with TBS (pH 7.6) for 5 minutes in room temperature and repeated three times. Data on this lectin can be found in [36–38]. Finally, the slides were counterstained with Harris-modified hematoxylin (Fisher Scientific, Hampton, NH) for increased visualization. Annotation was done digitally using Aperio ImageScope (Leica Biosystems, Buffalo Grove, IL) for positive pixel signal algorithm based on lectin staining.

Statistical analysis:

For all peaks (m/z values) mean intensity values were determined for each individual TMA spot. To facilitate statistical analysis, original data was transformed by log based on 10. Further descriptive statistics and statistical inference are all based on the log-transformed data.

To compare difference of glycan between HCC tissues and cirrhotic tissue, we applied t-test or Wilcoxon rank sum test, appropriately on data distribution. For tumor tissue and its adjacent tissue comparison, paired t-test or Wilcoxon rank sum test was also selected based on glycan data distribution. Two-sided hypothesis test was selected, p-value less than 0.05 was considered statistically significant. Receiver Operator Curves (ROC) curves were

constructed, area under curve (AUC) was considered as discriminant ability, standard error of AUC was derived from bootstrap.

For survival analysis, the median of the specific glycan was used as a cutoff line to classify patients who were above the median as being in the high group and the rest as the low group (choosing mean as cutoff derived similar results, because the mean and median were similar in 3 concerned glycans). We plotted the Kaplan-Meier survival curves of the high and low group, and log-rank test was applied to check survival difference between two groups. The Cox Proportional hazard model was used for further analysis.

Results:

Increased levels of branched and fucosylated N-linked glycans in HCC tissue.

We, and others, have previously correlated changes in glycosylation in the serum of individuals with the development of HCC [14–23] [2, 39–47]. To determine the glycan changes that occur directly in HCC tissue we utilized a MALDI based glycan imaging methodology [26] to examine the N-linked glycans that alter with the development of HCC. In our initial experiments, we examined five sections of HCC tissue obtained from patients with hepatitis B virus (HBV) - associated HCC, three sections of normal tissue and three sections of cirrhotic tissue. Supplementary Figure S1 shows the workflow of the tissue analysis and Figure 1 shows the results of a representative normal, cirrhotic and HCC tissue. In this figure, specific N-linked glycans are shown and their relative abundance presented via a heat map of individual glycan intensities across each tissue, where blue is low abundance and red is high abundance. Supplementary Figure S2 shows the same sections stained with hematoxylin and eosin staining in both a 1× and 10× magnification confirming diagnosis. In Figure 1, three N-linked glycans that were found in all tissues and three N-linked glycans that were elevated in the HCC as compared to the normal or cirrhotic tissue are presented. As Figure 1A-C shows, high mannose glycans such as Man 7, Man 8 (Figures 1A&B) and simple bi-antennary type glycans without substantial fucosylation (Figure 1C) can be found equally in normal, cirrhotic and HCC tissue. In contrast, glycans such as a tetra-antennary glycan without fucosylation (Figure 1D) or tetra-antennary glycan with single (Figure 1E) and multiple fucose residues (Figure 1F) are found predominantly in the HCC tissue as compared to the normal or cirrhotic tissue. It is noted that these mass values (glycan) were not observed without the application of PNGase F (data not shown).

Analysis in human liver TMA set

Machine and time restraints limited the number of whole tissue sections that could be examined and thus, to determine whether these N-glycan changes seen in the HCC tissue could be observed in a larger set of tissue samples, we examined two independent tissue microarrays (TMAs), one consisting of 48 HCC, 22 cirrhotic, and 5 normal tissue cases and another TMA consisting of 90 samples with HCC and 90 tissue samples of the adjacent untransformed tissue. Clinical and patient information for these samples are provided in Tables S1 and S2. Figure 2 shows both TMAs, demonstrating the imaging data received from the workflow. Supplementary Table S3 presents a list of all the potential glycans found in both of these TMAs. Elevations in specific glycans was determined by examining the

mean intensity values of each glycan structure in the HCC tissue and in the cirrhotic tissue for TMA #1 or the un-transformed adjacent tissue in TMA#2. A mean intensity value increase of 1.5-fold in the HCC sample as compared to the appropriate control tissue was considered elevated. Similarly, if the intensity was 0.5 times that of the appropriate control tissue, the structure levels were decreased. Supplemental Figure S4 highlights the observation that many of the 61 observed glycans were seen in 20–40% of the HCC tissue samples and often in less than 5% of the control tissue.

Two glycans that were observed as elevated in over 50% of the TMA samples, were a tetra-antennary glycan (glycan #15 in Supplemental Figure S4) and a tetra-antennary glycan with two fucose residues (glycan #16 in Supplemental Figure S4). As this family of glycan - tetra-antennary glycan with and without fucosylation - were observed in many of the samples, we further examined the level of this family of glycans in the TMAs. The level of the tetra-antennary glycan lacking fucose (Figures 2A), the tetra-antennary glycan with a single (Figure 2B) and double fucose residues (Figure 2C) in both the TMAs are shown. As before, darker red colors represent a higher intensity for the specific glycan while more blue tones represent less intensity. The mean values of signal intensities for specific glycans found in the HCC tissue as compared to the cirrhotic tissue (in TMA#1) or between the HCC tissue and the adjacent non-transformed tissue (TMA#2) were compared. Table 1 presents glycans (selected by lowest p value) that were significantly elevated ($p < 0.05$) in the HCC tissue as compared to the cirrhotic (TMA #1) or adjacent non-transformed tissue (TMA #2) as well as a glycan that was not altered in the HCC tissue. A master list of all N-glycan m/z values is provided in the supplementary data (Supplementary Table S3). As Table 1 shows, nine out of ten glycans that were elevated in the HCC tissue were fucosylated glycan. The glycans with the lowest p value were tetra-antennary glycans, with or without fucosylation. To further explore the branched and fucosylated glycome in these two TMAs, we examined the five observed tetra-antennary glycan that were altered in the TMA's by scatter plot and by AUROC analysis. Figure 3 A-E shows these data for TMA #1 and Figure 3 F-J shows these data for TMA #2. As Figure 3 shows, alterations in specific tetra-antennary glycoforms could be observed in both TMAs. For example, a glycan at m/z 2685.969, proposed as a di-fucosylated tetra-antennary glycan, was elevated in HCC tissue in both TMA#1 and TMA#2 (Figures 3B and 3G). Similarly, the tetra-antennary glycan (m/z 2393.840) devoid of fucosylation was also altered in both TMAs (Figure 3D and 3I). In contrast, TMA#1 had greater alterations in a tetra-antennary glycan with three fucose residues, as compared to TMA#2 (Figure 3A and F). Other versions of the tetra-antennary glycan family were also observed in both TMAs (Figure 3C and 3E).

Increased fucosylation seen by MALDI-MSI was further confirmed by lectin histochemistry. Supplementary Figure S3 shows the lectin histochemistry staining for one of the TMAs using a recombinant *Aleuria aurantia* lectin (AALN224Q) lectin which has enhanced binding to branched and core alpha 1,6 lined fucosylated glycan and reduced binding to alpha 1,2 linked fucose [48, 49]. Supplementary Figure S3, shows a side by side comparison of the lectin histochemistry and the MALDI imaging for one of the most prominent fucosylated glycan (m/z 2685.969; see Table 1), supporting the classification as fucosylated structures.

As fucosylation was a prominent feature of the altered glycan in HCC, with 33/61 of the proposed glycan structures containing fucose, fucosylation levels were further explored. To accurately determine the elevated levels of the fucosylated glycans seen between HCC and adjacent tissue, we examined the difference between the adjacent and HCC tissue of each individual patient in the matched tissue set TMA and determined the percentage of patients with elevated levels of each of these glycans in both their HCC tissue and their matched normal adjacent tissue. Elevation was again determined by using mean intensity values of these glycans in both the HCC and non-transformed adjacent tissue and if the value was 1.5 times that of normal levels, the patient was considered to have elevated levels of that glycan. As Figure 4 shows, 96% of patients had increased levels of at least one fucosylated structure. Those patients were then categorized into the number of these highly branched and/or fucosylated structures they were presenting, with patients demonstrating increased levels in anywhere from one fucosylated structure to all 33 found within the TMA.

Association of specific glycans with survival:

For the patient matched TMA (TMA#2), survival data were available allowing for the determination of an association between glycan and outcome. This was done for the three major glycans observed in the HCC tissue: a tetra-antennary glycan (m/z 2393.840), a tetra-antennary glycan with a single fucose (m/z 2539.957) and a tetra-antennary glycan with two fucose residues (m/z 2685.969). Patients with glycan expression greater than the median level in all tissue (both tumor and adjacent normal) were classified as high. There was no association with these glycans between the levels observed in the normal adjacent tissue and patient outcome (data not shown). In addition, as Figure 5A shows, the level of the m/z 2393.840 glycan was also not associated with patient outcome. The mean time of survival was 29 months in those with high or low levels of the m/z 2393.840 glycan. In contrast, as Figures 5B and 5C show, patients with high levels of the m/z 2539.957 or m/z 2685.969 glycans were associated with shorter survival times. For the m/z 2539.957 glycan, those with high levels had a median survival time of 25 months, while patients with low levels of this glycan had a median survival time of 35 months. Similarly, for the m/z 2685.969 glycan, those with high levels had a median survival time of 25 months, while patients with low levels of this glycan had a median survival time of 32 months. Cox proportional hazard model analysis showed patients with one unit increase of the m/z 2685.969 glycan would enhance the hazard(risk) about 3-fold, $p=0.0334$. One unit increase of the m/z 2539.957 glycan would increase hazard about 8fold, $p=0.0078$. There was no association between these glycans and stage or grade of HCC (data not shown).

Discussion:

Alterations in glycosylation have been long observed with HCC[50–55]. Much of this work was shown in serum, with little analysis directly in the HCC tissue itself or has been analyzed following dissection of tissue and the mixing of the multiple hepatic (and non-) cell types. Here we utilized MALDI-glycan imaging to identify the glycans that occur directly in 138 HCC patient tissue samples. In our analysis of both TMA's, there were 61 glycans that were found to be upregulated in at least one HCC tissue sample (See supplementary Table S3). In addition, there were 10 glycans that were significantly ($p<0.05$) increased in at least

30% of the HCC tissue samples as compared to either cirrhotic or adjacent tissue (see Supplementary Figure S4).

Our previous MALDI glycan imaging developmental work had utilized a small number of HCC tissue samples and a 16 patient HCC TMA[25]. In that study, alterations in both branching and fucosylation were observed but the sample size was too small to determine the significance of the changes detected. Here, we have extended that work through an analysis of a larger number of samples and also with the association with outcome data regarding the observed glycans.

Surprisingly, only two major classes of glycan were observed in HCC tissue as compared to either cirrhotic tissue or adjacent untransformed tissue. The first was a tetra-antennary glycan structures and the second was an increase in the level of fucosylation. It is noted that the tetra-antennary glycan was only observed in HCC tissue and not in adjacent tissue or cirrhotic tissue. The tetra-antennary glycan is formed through the action of alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A (MGAT5), which has been associated with many cancers through mutations of the telomerase reverse transcriptase (hTERT) [56] and through activation of the Ras/Raf pathway [57].

The second major alteration observed in the HCC tissue was increased fucosylation. This glycan change has been observed in the serum for many years, but a clear understanding of where this material derives was not known. However, glycan analysis of tumor derived material was unable to identify fucosylation as being increased in HCC [58, 59]. This was most likely the result of the method used, which involved homogenization of tissue and mixing of cell types. In contrast, by using the MALDI glycan imaging method we were able to observe increased levels of fucosylation on independent sample sets. Most often on tetra-antennary glycan but also to a lesser extent on bi-antennary and tri-antennary glycan. Indeed, there is now significant evidence that transformed hepatocytes are the source cells for serum fucosylated proteins. Recent work showed that as hepatocytes undergo an epithelial–mesenchymal transition (EMT), they increase the genes, such as alpha-1,6-fucosyltransferase gene (*FUT8*), which are involved in fucosylation [60]. This is consistent with lung cancer, where *FUT8* increased as a direct result of an EMT [61]. In addition, a recent report has indicated that HCC downregulates miR-122 and leads to the upregulation of *FUT8*[62]. It is also noted that the deletion of *FUT8* in a mouse model inhibits chemical induced HCC by the down regulation of cancer associated signaling pathways [63, 64]. Together, this data suggests very strongly that fucosylation originates from the cancer itself and prior analytical glycan tools were not able to detect this change within the tumor. In addition, over 95% of the HCC samples analyzed had increased levels of one or more fucosylated glycan, while normal adjacent tissue did not, supporting the hypothesis that fucosylation is an event associated with the general act of cellular transformation.

A drawback of the MALDI imaging mass spectrometry approach is the inability to differentiate the anomeric linkages of each fucose, and thus, we are unsure if the fucose modification is core alpha-1,6-linked fucosylation or outer arm fucosylation. Both fucose linkages have been observed in the serum of those with HCC and both may be increased in

the HCC tissue. The lectin staining does support the hypothesis that fucosylation is occurring in the HCC tissue.

In addition, we observed only a few sialylated structures by MALDI-glycan imaging and it is possible that these 1) were not detected by our method or 2) we had sialic acid loss following ionization. It is also highly likely that both things are true and methods to stabilize sialic acids will be required for analysis of these structures[29].

While the identity of the proteins containing these changes are unknown, several proteins have been characterized as containing the glycans shown to be up-regulated in HCC tissue. For example, we have recently shown that low molecular weight (LWM) kininogen contains fucosylated tetra-antennary glycan and that this protein can act as a serum biomarker of HCC [65]. Additionally, serum fucosylated haptoglobin and fucosylated fibronectin have been shown to contain branched fucosylated tetra-antennary glycan in HCC [44].

Lastly, heterogeneity was observed in the glycans associated with HCC and it is assumed that this most likely is the result of the underlying genetic heterogeneity found with the disease[62]. In conclusion, we have shown that two major glycan changes are associated with HCC, increased branching and increased fucosylation. Hopefully, in the future, these glycan changes can be exploited for the early detection of HCC and potentially in the treatment of HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Detection of various N-linked glycans in normal, cirrhotic and HCC tissues.

While certain glycans are found in all tissues (A-C), some glycans are found predominantly in the HCC tissue (C-E). Images were acquired with 150 μm raster step size on a Bruker 7T solariX XR ICR FTMS system. Ion intensities are normalized to the TIC of each ion across the tissue. Color scale bars are included and autocorrected for the range of intensities plotted. For glycans, red triangle, fucose; blue square, N-acetylglucosamine; green circles, mannose; yellow circles, galactose.

Figure 2. Representative imaging data from both TMA datasets.

Representative image data collected for both TMA sets (first TMA with independent samples is on top with the second TMA with HCC and adjacent tissue is on bottom) showing three different glycan structures: A) 2393.840 m/z, B) 2539.957 m/z and C) 2685.969 m/z. For the first TMA, the HCC tissue is indicated at top with the cirrhotic samples in the middle and the healthy tissue samples in the box at the bottom right corner. For the second TMA, the + above each column represents the HCC tissue and the consecutive – column represents the matched normal adjacent tissue section. The proposed glycan is presented at the bottom of each panel

Figure 3. Analysis of human liver TMA datasets.

Proposed glycan structure, log transformed intensity scatter plot with the red diamond indicating mean and associated p-value, and Receiver Operating Characteristic (ROC) curve with AUC value for select structures. In panels A-E, analysis was done comparing HCC versus cirrhotic samples. A, B, C, and E utilized a student t-test for their p-value while D utilized a Wilcoxon Rank Sum Test. For Panels F-J, analysis was done comparing HCC versus adjacent tissue. F, G, H, and J utilized a paired t-test for their p-value while panel I utilized a Wilcoxon Signed Rank Test.

Figure 4. Patients demonstrating elevated levels of fucosylated glycan structures.

A total of 33 fucosylated glycans were found elevated in the patient-matched TMA. Comparing HCC to the un-transformed adjacent tissue, a 1.5 $\times$ relative intensity increase in HCC tissue was used to classify patients as elevated. Of the 89 patients able to be analyzed, 96% (85 patients) demonstrated elevated levels of at least one of these fucosylated structures (left). Of these 85 patients, they were further classified into varying classes based on the number of fucosylated structures they had elevated levels for. 27% (23 patients) had elevated levels of one to four fucosylated structures, 25% (21 patients) had elevated levels off five to eight structures, with 8% of patients showing elevated levels of 19 or more of these fucosylated structures with one patient showing elevated levels all 33 fucosylated structures found.

Figure 5. Survival Plots for Branched and Fucosylated Glycans:
Kaplan–Meier Survival plots for glycan at m/z 2393.840 (A) 2539.957 (B) and 2685.968 (C) from the 90 patient TMA. Survival time is in months. See text for more detail.

Table 1.

Glycans altered in HCC versus Cirrhosis or HCC versus adjacent tissue.

Observed m/z ¹	Proposed Glycan Structure ²	Composition ³	<i>P</i> value TMA 1 ⁴	<i>P</i> value TMA 2 ⁵
2832.046		Hex7dHex3HexNAc6 + 1Na	0.00015	0.2105
2685.969		Hex7dHex2HexNAc6 + 1Na	1.23e-07	7.59e-15
2539.957		Hex7dHex1HexNAc6 + 1Na	0.00016	0.02739
2466.895		Hex6dHex3HexNAc5 + 1Na	0.0195	0.002727
2465.878		Hex6dHex1HexNAc5NeuAc1 + 1Na	0.2427	0.000654
2393.840		Hex7HexNAc6 + 1Na	3.11e-08	9.33e-16
2377.889		Hex6dHex1HexNAc6 + 1Na	2.98e-08	6.71e-11
2174.806		Hex6dHex1HexNAc5 + 1Na	0.03208	0.3902
2012.724		Hex5dHex1HexNAc5 + 1Na	0.000281	0.000105
1850.656		Hex4dHex1HexNAc5 + 1Na	0.000103	1.71e-09
1647.589		Hex4dHex1HexNAc4 + 1Na	0.00633	0.00571
1954.689		Hex5HexNAc4NeuAc1 + 1Na	0.63172	0.2382

Observed m/z ¹	Proposed Glycan Structure ²	Composition ³	<i>P</i> value TMA 1 ⁴	<i>P</i> value TMA 2 ⁵
1663.567		Hex5HexNAc4 + 1Na	0.8752	0.0012

¹) Observed mass to charge ratio value

²) The proposed glycan structure based upon the m/z value.

³) The composition of the identified M/Z value.

⁴) *P* value comparing the HCC to cirrhotic tissue. Analysis by students T-test.

⁵) *P* value comparing the HCC to adjacent tissue.