

## Increased Levels of Tetra-antennary N-Linked Glycan but Not Core Fucosylation Are Associated with Hepatocellular Carcinoma Tissue

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### Abstract

**Background:** Alterations in glycosylation have long been associated with the development of cancer. In the case of primary hepatocellular carcinoma (HCC), one alteration that has often been associated is increased amounts of fucose attached to the N-glycans of serum proteins secreted by the liver.

**Methods:** In an effort to determine the origin of this increased fucosylation, we have conducted N-linked glycan analysis of HCC tissue, the surrounding nontumor tissue, and compared this to tissue from a nondiseased adult liver.

**Results:** Surprisingly, no difference in the level of fucosylation was observed from the three donor groups, suggesting that the increased levels of fucosylation observed in serum of those with HCC is not the result of increased synthesis of fucosylated proteins in the cancer tissue. On the other hand, increased levels of a tetra-antennary glycan were observed in the HCC tissue as compared with the surrounding tissue or to the nondiseased livers.

**Conclusions:** This represents, to our knowledge, one of the first reports associating increased levels of branching with the development of HCC.

**Impact:** The identification of increased levels of tetra-antennary glycan on liver tumor tissue, as opposed to adjacent or nondiseased tissue may lead to improved detection of HCC. *Cancer Epidemiol Biomarkers Prev*; 21(6); 925–33. ©2012 AACR.

### Introduction

Infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is the major etiology of hepatocellular carcinoma (HCC; refs. 1–4). Both HBV and HCV cause acute and chronic liver infections, and most chronically infected individuals remain asymptomatic for many years (5). Between 10% and 40% of all chronic HBV carriers eventually develop primary liver cancer (HCC), and it is estimated that more than one million people worldwide die of HBV/HCV-associated liver cancer (2, 6, 7). Indeed, HBV and HCV infections are associated with more than

80% of all HCC cases worldwide and can be as high as 96% in regions where these viruses are endemic (3).

The progression from liver disease to liver cancer is often monitored with serum levels of oncofetal glycoprotein,  $\alpha$ -fetoprotein (AFP), or the core fucosylated glycoform of AFP (AFP-L3; refs. 8–10). However, AFP can be produced under circumstances other than HCC, including association with other liver diseases (8–10), and it is not elevated in all patients with HCC (11). Therefore, the reliability of AFP levels as a screening tool for HCC has been questioned (12), and more sensitive serum biomarkers for HCC are desired.

Using various proteomic methods to look for biomarkers useful in the early detection of HCC, we identified changes in the levels of N-glycan in total serum and in serum depleted of IgG and/or major acute-phase proteins (13–15). The change in glycosylation observed in the serum associated with HCC was an increase in the level of fucosylation, which has also been reported by others. In this study, we expanded our search by analyzing the glycosylation of HCC tissue as compared with the non-cancerous adjacent tissue. In addition, we have examined the N-linked glycosylation of normal healthy livers. Surprisingly, increased levels of fucosylation were not observed in the HCC tissue as compared with either surrounding tissue or "healthy" tissue. Thus, the increased

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**Table 1.** Patient information

Patient no. <sup>a</sup>	Age/gender <sup>b</sup>	Etiology <sup>c</sup>	T-BIL <sup>d</sup>	AST <sup>e</sup>	ALT <sup>f</sup>	GGTP <sup>g</sup>	AFP <sup>h</sup>
1	69/M	HCV	1.2	142	286	60	12
2	63/M	HCV	0.7	77	73	47	143
3	69/F	HCV	0.8	64	57	103	53/103
4	59/M	HCV	0.5	83	101	121	24
5	59/M	HCV	0.8	102	115	235	12
6	66/M	HCV	0.8	99	206	106	40
7	63/M	HBV	1.4	58	53	180	17
8	65/F	HCV	0.9	35	37	77	13
9	63/F	HBV	0.9	57	47	40	6
10	63/M	HCV	0.5	48	54	53	15
11	53/M	HCV	0.7	157	154	179	96
12	64/M	HCV	0.9	133	141	57	96
13	64/M	NBNC	0.4	48	69	171	12
14	66/F	HBV	1.3	19	14	14	1,276
15	50/F	HBV	NA	38	51	NA	18,500
16	NA	NA	NA	NA	NA	NA	NA

Abbreviation: NBNC, non-HBV and non-HCV liver disease.

<sup>a</sup>Patient pairs. For each patient, HCC and adjacent tissue was obtained.

<sup>b</sup>The gender, male (M) or female (F), and age of the patient.

<sup>c</sup>The etiology of the cancer.

<sup>d</sup>Total bilirubin levels in mg/dL.

<sup>e</sup>Aspartate transaminase in IU/L.

<sup>f</sup>Alanine transaminase in IU/L.

<sup>g</sup>Gamma-glutamyl transpeptidase in IU/L.

<sup>h</sup>AFP in ng/mL.

levels of fucosylation that are observed in sera of patients with HCC, but not in those of benign liver diseases, suggests an abnormal secretion of fucosylated proteins in HCC. In contrast, increased levels of tetra-antennary glycan were observed in the HCC tissue as compared with both the surrounding tissue and the tissue from a normal liver. Tetra-antennary glycans result from increased activity of the *N*-acetylglucosaminyltransferase V (GnT-V) enzyme, which has long been associated with cancer development and metastatic potential (16–19). This is the first report of increased levels of tetra-antennary glycan in HCC tissue. Importantly, this change was found in both AFP-positive and more importantly, AFP-negative tumors, indicating its potential role as a cancer biomarker.

## Materials and Methods

### Patients

The present study enrolled 16 patients of ages  $62.4 \pm 4.2$  (10 males, 5 females, and 1 NA) with HCC who had undergone surgical resection between January 1992 and December 1997 at Osaka Rosai Hospital (Nakasonecho, Kitaku, Sakai, Japan). For all patients, hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), and HCV antibody were routinely examined by commercially available methods. Positivity for HCV-RNA was confirmed in a subset of patients with HCV. Of the 16

patients enrolled, HBsAg and HCV antibody were positive in 4 and 10 patients, respectively. One patient was double negative for HBV and HCV and another patient had no clinical information. Clinical data of each patients are described in Table 1. All patients with HCC had no history of treatment for HCC before the operation in which liver samples were collected. Tumor samples and nontumor portions of the liver were obtained during the surgical resection and were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until used. Nontumor tissues were collected at a distance of at least 5 cm from the cancer lesions. All of the liver samples were histologically examined by an experienced pathologist who had no knowledge of the analytic results. This project was approved by the Ethics Committees of Osaka Rosai Hospital hospitals and written informed consent was obtained from patients in this study.

### Tissue preparation

Tissue was sliced into fine pieces and dounce homogenized in a 30 mmol/L Tris-HCl pH 7.4 buffer containing 0.3% SDS, 3% dithiothreitol (DTT), 4 mmol/L MgCl<sub>2</sub>, and a Protease Inhibitor Cocktail (Roche) and placed on ice for 1 hour. The lysate was spun down at 14,000 rpm for 30 minutes and buffer exchanged into PBS at pH 7.2. Protein concentration was assayed using a NanoDrop (ThermoFisher).

### Glycan analysis

Four hundred micrograms of protein lysate or 5  $\mu$ l of human serum was absorbed into a 3 mm dehydrated gel plugs. The protein gel plugs were then reduced by boiling in 35 mmol/L DTT for 5 minutes, and alkylated at room temperature in 100 mmol/L iodoacetamide. The gel plugs were fixed, 3  $\times$  30 minutes in a solution of 40% methanol and 7% acetic acid. A washing step is conducted by dehydration in acetonitrile, rehydration in 20 mmol/L ammonium bicarbonate, and dehydration with acetonitrile, then dried in a speedVac. Ten microunits of peptide:*N*-glycosidase F (Prozyme) was diluted with 7  $\mu$ l of 20 mmol/L ammonium bicarbonate, pH 7, and allowed to adsorb into the gel plug. The gel plug was then covered with buffer and allowed to incubate overnight at 37°C. The glycans were eluted from the gel plug by sonication in Milli-Q water 3 times; the pooled elutant was dried down and labeled with a 2-aminobenzamide dye (Ludger) according to the manufacturer's instructions. Excess dye was removed with paper chromatography and the glycans were passed through a 0.22- $\mu$ m syringe filter. Fluorescently labeled glycans were subsequently analyzed by high-performance liquid chromatography (HPLC) using a TSK-Gel Amide 80 column (Tosoh). The mobile phase consisted of solvent A (50 mmol/L ammonium formate, pH 4.4) and solvent B (acetonitrile). The gradient used was as follows: linear gradient from 20% to 58% solvent A at 0.4 mL/min for 152 minutes followed by a linear gradient from 58% to 100% solvent A for the next 3 minutes. The flow rate was increased to 1.0 mL/min, and the column was washed in 100% solvent A for 5 minutes. Following the washing step, the column was equilibrated in 20% solvent A for 22 minutes in preparation for the next sample run. HPLC analysis was conducted with the Waters Alliance HPLC System, complemented with a Waters fluorescence detector, and quantified with the Millennium Chromatography Manager (Waters Corporation). Glycan structures were identified by calculating the glucose unit value and exoglycosidase digestion, as previously described (20).

### Statistical analysis

Descriptive statistics for patients were compared by scatter plots that included the outliers. All values were reported as mean values  $\pm$  SD unless otherwise stated. Because the data did not follow a typical Gaussian distribution, a nonparametric test (2-tailed, 95% confidence interval, Mann-Whitney *U* test) was used to determine statistical differences between groups. To determine the optimal cutoff value for each marker, the receiver operating characteristic (ROC) curves were constructed using all possible cutoff values for each assay. The area under the ROC (AUROC) curves were constructed and compared as described previously. A 2-tailed *P* value of 0.05 was used to determine statistical significance. All descriptive analyses were conducted using a GraphPad Prism. For combinatorial analysis, AFP values were log transformed to bring the values of all markers into a similar scale, and a centering and scaling approach was taken to

normalize data before analysis using a multivariate logistic regression method, using the R package, version 2.8.1.

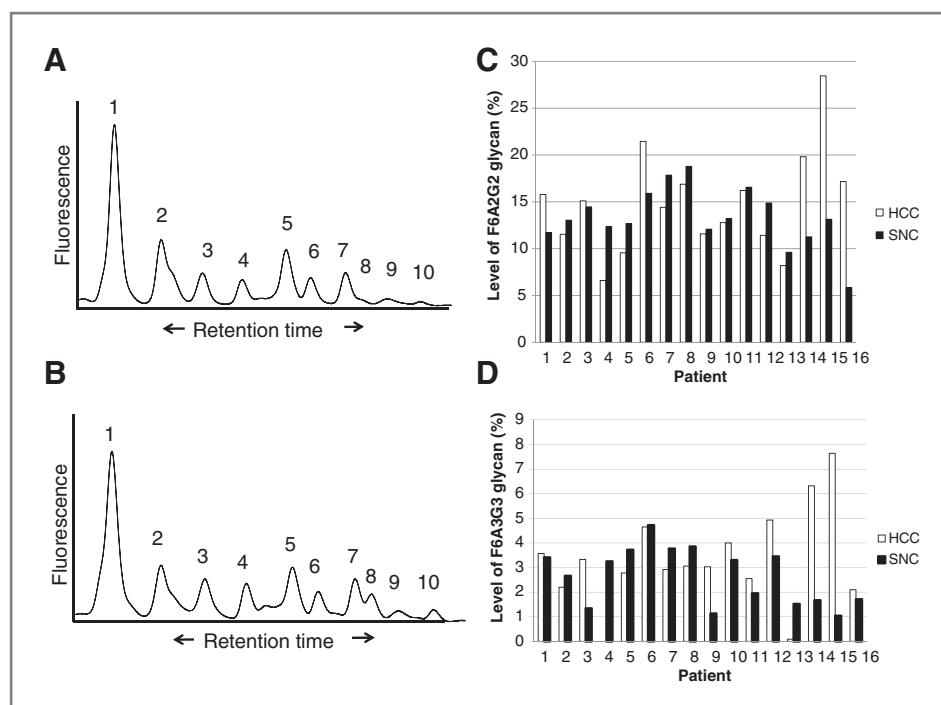
### Results

#### Fucosylation is not increased in HCC tissue as compared with adjacent or control tissue

In previous work, we and others, have observed increased levels of core fucosylated glycan in the serum of patients with HCC (13–15, 21–25). In an effort to determine whether the cancerous tissue is the source of the increased level of serum core fucosylation we have conducted *N*-linked glycan analysis of normal, tumor, and adjacent nontumor (fibrotic) tissue. Sixteen tumor samples, along with adjacent tissue obtained from patients undergoing tumor resection are described in Table 1. In addition, "normal, healthy" tissue was obtained from 3 independent "control livers" from commercial sources. For all tissue, total protein lysates were made and the amount of total protein was quantified before the *N*-linked glycans attached to total protein were removed enzymatically using peptide:*N*-glycosidase F and labeled with a fluorescent dye before analysis of the *N*-linked glycans via sequential exoglycosidase digestion (26–28).

Figure 1A and B shows the simplified desialylated glycoprofile for a representative patient set (HCC and surrounding tissue) following treatment with neuraminidase (*Arthrobacter ureafaciens*). Each peak corresponds to a different glycan structure (or multiple structures) and as this figure shows, little difference is observed between the adjacent and HCC tissue from the individual patient shown. Sequential exoglycosidase digestion (data not shown) was used to identify the core fucosylated *N*-linked glycan. Two major types of core ( $\alpha$ -1,6 linked) fucosylated glycan were observed in the liver tissue, a core fucosylated biantennary glycan and a core fucosylated triantennary glycan. The levels of each of these structures in the adjacent and HCC tissue are shown in Fig. 1C and D. As this figure shows, the level of core fucosylation is not substantially increased in the patients with HCC as compared with the adjacent tissue or normal tissue from a donor liver. Figure 1C shows the relative levels of the core fucosylated biantennary glycan in all 16 tissue pairs. As summarized in Table 2, the core fucosylated biantennary peak had a mean value of 13.34% of the total glycan pool in the adjacent tissue, and 14.81% in the HCC tissue. This compares with an average of 11.6% in the control tissue (Table 3). None of these differences were statistically different. Pairwise statistical analysis was also used to determine whether difference in core fucosylation could be observed between patient pairs; however, no statistically significant differences could be observed.

As Fig. 1A and B highlight, a core fucosylated triantennary glycan was also observed (peak 5). The levels of this glycan in the 16 tissue pairs is shown in Fig. 1D and similar to the results observed in Fig. 1C, the levels of this core fucosylated species are not consistently altered in the



**Figure 1.** N-Linked glycosylation of total protein from representative HCC tissue and adjacent liver tissue. Desialylated N-linked glycan profile of representative adjacent liver tissue (A) or HCC tissue (B) from one matched pair. Identified species are indicated as follows, using terminology described elsewhere (45): peak 1, A2G2; peak 2, F(6)A2G2; peak 3, M7; peak 4, A3G3; peak 5, comigrating M8 and F(6)A3G3; peak 7, M9; peak 8, A4G4; peak 9, F(6)A4G4. Peaks 6 and 10 are incompletely characterized, although peak 6 contains one or more structures containing outer arm fucose. C, the level of the F(6)A2G2 glycan in the matched pairs of tissue. The percentage of the F(6)A2G2 glycan as a function of the total glycan profile is shown. D, the level of the F(6)A3G3 glycan in the matched pairs of tissue. As with (C), the percentage of the F(6)A3G3 glycan is a function of the total glycan profile. For Figures C and D, HCC, hepatocellular carcinoma tissue; SNC, surrounding non cancer tissue.

HCC tissue as compared with the adjacent tissue. As summarized in Table 2, the core fucosylated triantennary peak had a mean value of 2.66% of the total glycan pool in the adjacent tissue, and 3.32% in the HCC tissue. This compares with an average of 2.9% in the control tissue (Table 3). These numbers were not statistically different ( $P > 0.05$ ).

#### Increased branching is observed in HCC tissue

In contrast to core fucosylation, which was not consistently altered in the HCC tissue, as compared with the adjacent tissue, increased levels of tetra-antennary glycan (A4G4) were observed in the HCCs when compared with the adjacent tissue. Figure 2 shows a focus of the tetra-

antennary N-linked glycan following treatment with neuraminidase (*Arthrobacter ureafaciens*) and  $\alpha$ -(1-2,3,6)-jack bean mannosidase (Fig. 2A). Subsequently, the peak preliminarily identified as a A4G4 glycan was collected and digested individually with almond meal and bovine kidney fucosidase (for alpha 1-3,4,6-linked fucose; Fig. 2B) and bovine jack bean  $\beta$ -(1-4,6) galactosidase (Fig. 2C). As Fig. 2 shows, whereas treatment with the fucosidases did not alter this peak (Fig. 2B), indicating that this peak is not fucosylated, treatment with the beta-galactosidase shifted the peak to a level consistent with an A4G0 glycan (an agalactosylated tetra-antennary glycan). Digestion of this structure with hexosaminidase (Glyko;  $\beta$ -N-Acetyl-hexosaminidase/HEXase I) digested this structure to the

**Table 2.** Fucosylated and corresponding nonfucosylated glycan structures associated with HCC and adjacent tissue

Glycan <sup>a</sup>	Tumor mean <sup>b</sup> ( $\pm$ SD)	Nontumor mean <sup>c</sup> ( $\pm$ SD)	P
A2G2	37.93 ( $\pm$ 4.70)	38.25 (1.95)	>0.05
F(6)A2G2	14.81 ( $\pm$ 5.44)	13.34 ( $\pm$ 3.17)	>0.05
A3G3	7.06 ( $\pm$ 2.42)	6.40 ( $\pm$ 1.74)	>0.05
F(6)A3G3	3.32 ( $\pm$ 1.96)	2.66 ( $\pm$ 1.16)	>0.05
A4G4	2.87 ( $\pm$ 1.03)	1.55 ( $\pm$ 0.65)	0.0003
F(6)A4G4	1.76 ( $\pm$ 0.84)	1.49 ( $\pm$ 0.65)	>0.05

Abbreviations: A2G2, biantennary glycan; A3G3, triantennary glycan; A4G4, tetra-antennary glycan; F(6)A2G2, core fucosylated biantennary glycan; F(6)A3G3, core fucosylated triantennary glycan; F(6)A4G4, core fucosylated tetra-antennary glycan.

<sup>a</sup>Key glycans identified in HCC and adjacent tissue.

<sup>b</sup>The mean percentage of each glycan in the total glycan profile of the tumor tissue.

<sup>c</sup>The mean percentage of each glycan in the total glycan profile of the adjacent (nontumor) tissue.

**Table 3.** N-linked glycan content of normal liver tissues

No <sup>a</sup>	Glycan <sup>b</sup>	Liver 1 <sup>c</sup>	Liver 2 <sup>d</sup>	Liver 3 <sup>e</sup>	Liver mean <sup>d</sup> ( $\pm$ SD)
1	A2G2	38.05	37.3	29.36	34.9 ( $\pm$ 4.8)
2	F(6)A2G2	11.45	10.15	13.19	11.6 ( $\pm$ 1.5)
3	M7	7.88	9.61	8.89	8.79 ( $\pm$ 0.87)
4	A3G3	5.75	5.91	4.83	5.5 ( $\pm$ 0.58)
5*	F(6)A3G3	1.95	3.91	2.87	2.9 ( $\pm$ 0.98)
5*	M7	7.88	9.61	8.89	8.79 ( $\pm$ 0.87)
7	M9	12.39	10.31	12.33	11.68 ( $\pm$ 1.2)
8	A4G4	2.13	1.45	1.20	1.59 ( $\pm$ 0.48)
9	F(6)A4G4	1.35	0.41	2.13	1.3 ( $\pm$ 0.86)

Abbreviations: A2G2, biantennary glycan; A3G3, triantennary glycan; A4G4, tetra-antennary glycan; F(6)A2G2, core fucosylated biantennary glycan; F(6)A3G3, core fucosylated triantennary glycan; F(6)A4G4, core fucosylated tetra-antennary glycan; M7, Mannose 7 glycan; M8, Mannose 8 glycan; M9, Mannose 9 glycan.

<sup>a</sup>The glycans as numbered in Fig. 1, asterisks indicate comigrating peaks that were subject to exoglycosidase digestion to quantify.

<sup>b</sup>Key glycans associated with tissue examined.

<sup>c</sup>The mean percentage of each glycan in the total glycan profile of the healthy liver tissue.

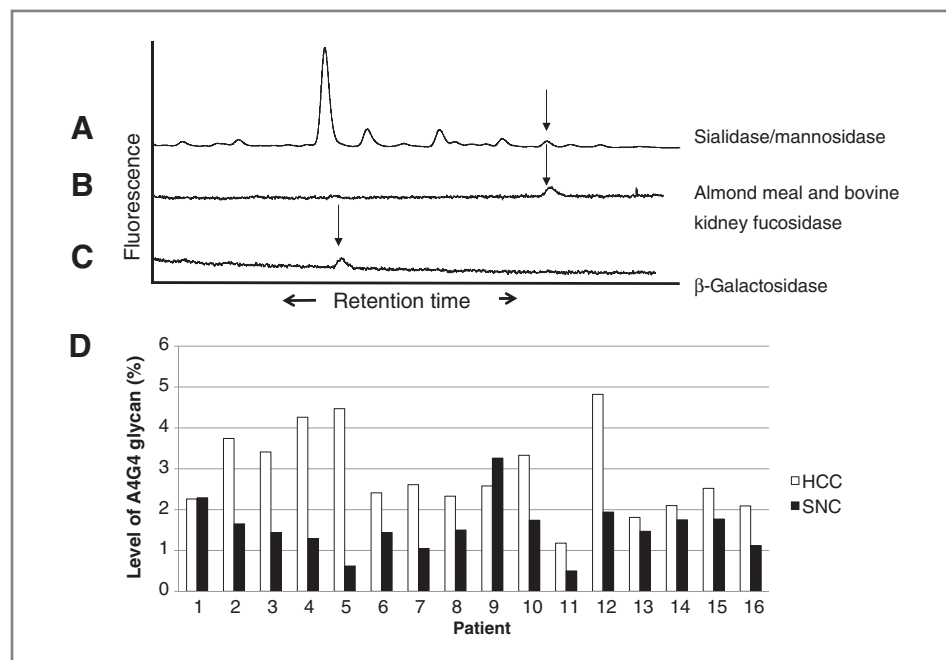
<sup>d</sup>The mean level of each glycan.

chitobiose core (M3N2), confirming that this peak consisted of a tetra-antennary N-linked glycan. The levels of the tetra-antennary N-linked glycan in the HCC and adjacent tissue are shown in Fig. 2D. As this figure shows, the level of the tetra-antennary glycan was increased in 14 of 16 HCC samples, as compared with the adjacent tissue. The levels of the tetra-antennary glycan in normal tissue were similar to what was observed in the adjacent tissue (see Tables 2 and 3). The complete profile of all 16 tissue pairs, with the A4G4 peak indicated are shown in Supplementary Fig. S1.

The mean level of the A4G4 glycan in the HCC tissue was 2.87% of the total glycan pool and 1.55% in the adjacent tissue. Although this is less than a 2-fold difference, 14 of 16 of the HCC tissues were elevated for the glycan relative to the matched nontumor samples, and the mean difference was statistically different ( $P = 0.0003$ ). The level of this glycan in normal liver was 1.59, very similar to the level observed in the nontumor tissue (Table 3).

In an effort to further confirm the results obtained by N-linked glycan sequencing, we conducted a lectin blot of total pooled protein from either the HCC or adjacent

**Figure 2.** Increased levels of tetra-antennary glycan are associated with HCC tissue. A focus on the tetra-antennary glycan showing (A4G4) the digestion with (A) sialidase (*Arthrobacter ureafaciens*) and jack bean mannosidase, (B) a mixture of bovine kidney (1,6) and almond meal  $\alpha$ -(1-3,4) fucosidases, and (C) jack bean  $\beta$ -(1-4,6) galactosidase. The A4G4 peak was collected after treatment with the mannosidase and digested individually. Treatment with jack bean  $\beta$ -(1-4,6) galactosidase results in the removal of the terminal galactose residues and the creation of an A4G0 glycan as indicated by the arrow. D, the level of the A4G4 glycan in the matched pairs of tissue. The percentage of the A4G4 glycan as a function of the total glycan profile is shown. For Figures C and D, HCC, hepatocellular carcinoma tissue; SNC, surrounding non cancer tissue.

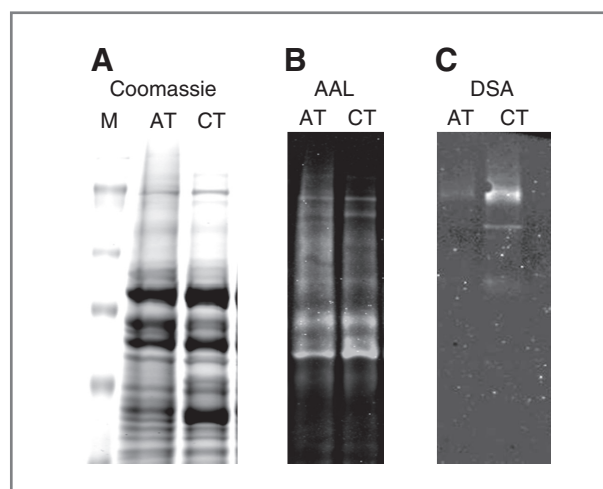


tissue. In Fig. 3B, the fucose binding *Aleuria aurantia* lectin (AAL) was used to determine the level of fucosylation in the HCC and the adjacent tissue. Consistent with the results obtained via *N*-linked glycan sequencing, the AAL lectin blot showed a similar banding pattern in the HCC tissue and in the adjacent tissue, indicating no major change in fucosylation in HCC tissue.

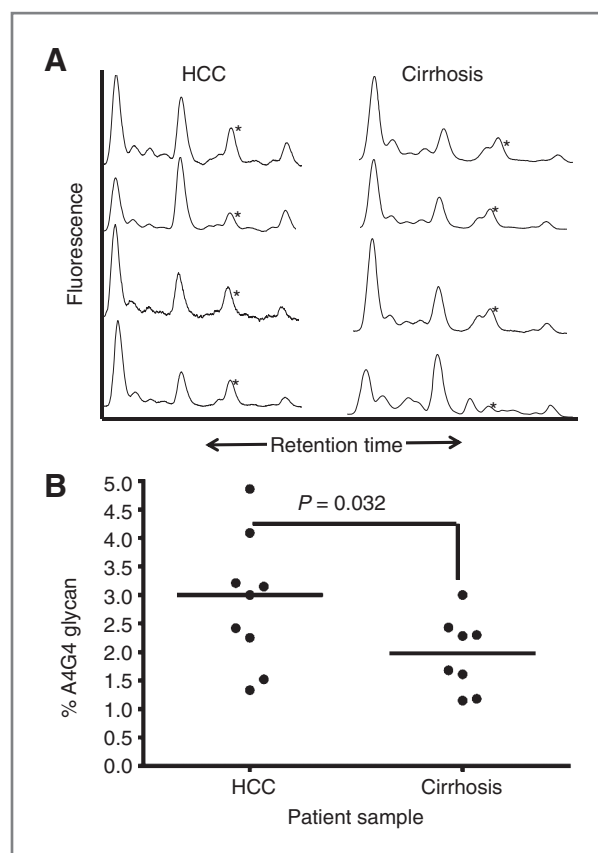
Changes in branching were analyzed using the *Datura stramonium* lectin (DSL), which has high affinity toward tri- and tetra-antennary complex-type oligosaccharides. As Fig. 3C shows, consistent with the results obtained in Fig. 2, there was substantially more binding of the DSL lectin with the HCC tissue, as compared with the adjacent tissue, suggesting increased levels of branching in the HCC tissue.

### Increased tetra-antennary glycan associated with serum glycoproteins in the serum of patient with HCC

In an effort to determine whether the increased level of branched *N*-linked observed in the tissue of patients with HCC could be seen in the serum of patients, we have conducted glycan analysis on serum from 9 HCC and 8 cirrhotic patients. The *N*-linked glycan profile, with a focus on the A4G4 glycan, for 4 representative HCC and 4 cirrhotic, patients is shown. As Fig. 4A shows, there is an increase in the level of the A4G4 glycan in the HCC samples as compared with the cirrhotic samples. Figure 4B shows the level of the A4G4 glycan in all 9 HCC and 8 cirrhotic samples. As this figure shows, the level of A4G4 glycan in the HCC samples ranged from 1.33% of the total glycan pool to 4.8% of the total glycan pool with a media value of 3.00 ( $\pm 1.13$ ). In the patients with cirrhosis the



**Figure 3.** Lectin blotting of HCC and adjacent tissue. Pooled HCC or adjacent tissue was examined by (A) Coomassie staining for total protein, (B) via the AAL for fucosylated proteins, or (C) the DSA for the detection of branched glycan. As this figure shows, consistent with the glycan data, whereas there is no difference in the level of fucosylation in the HCC and adjacent tissue (B) there is a significant difference in the level of branched glycan in the HCC tissue as compared with the adjacent tissue. M, markers; AT, adjacent tissue; and CT, cancer tissue.



**Figure 4.** Increased levels of branching on serum glycoproteins from patients with HCC. A, focus on larger branched glycans from 4 representative cirrhotics, and 4 representative HCC samples highlighting specific changes in glycosylation observed in HCC. The A4G4 peak is indicated with an asterisk. As in Fig. 2, sequential exoglycosidase digestion was used to identify *N*-linked glycan. B, scatter plot of the A4G4 glycan from 9 HCC and 8 cirrhotic patients showing the level of the A4G4 glycan in all patients. The solid line indicates the median level and the *P* value is indicated.

level of A4G4 glycan ranged from 1.0% of the total glycan pool to 3.0% of the total glycan pool with a median value of 1.98 ( $\pm 0.653$ ). Although the sample size is small, the difference was statistically significant ( $P = 0.032$ ).

### Discussion

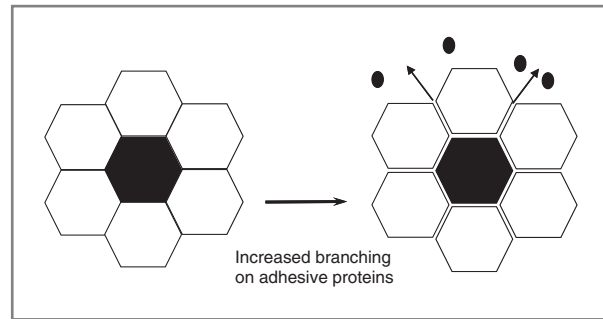
Changes in *N*-linked glycosylation on serum proteins have been associated by us and by others with liver cancer. The most prominent change has been the increased levels of  $\alpha$ -1,6- and  $\alpha$ -1,3-linked fucosylation (13–15, 21, 22, 29–35). In an effort to identify the source of this increase we have conducted *N*-linked glycosylation analysis of HCC tissue, surrounding tissue and liver tissue from nondiseased livers. Surprisingly, the normal, HCC, and tumor-adjacent tissue had similar levels of fucosylation, suggesting that the increase in liver-derived fucosylated proteins observed in the circulation is not the result of increased production from either the HCC tissue or the surrounding tissue. Previous reports have indicated that the level of the fucosyltransferases involved in core fucosylation were

similar in cancer and cirrhotic tissue (36). Our glycan analysis is consistent with this finding and extends it by showing that the levels of core fucosylation are similar in both diseased and healthy livers.

One hypothesis is that it is not the production of fucosylated proteins that is greater in HCC but rather increased secretion into the circulation that is responsible for the increased level of fucosylated proteins into the serum (37). The results presented here would support this hypothesis. Indeed, the level of core fucosylated glycan that was observed in liver tissue was much greater than that observed in the circulation of all groups, including individuals with no known liver disease. Interestingly, the level of fucosylation observed in the tissue was similar to what was observed in the serum of patients with HCC (>8.0%), further supporting the hypothesis that altered secretion is the main reason for increased level of fucosylated glycan in the serum of patients with HCC. It is noted that fucosylation changes could be specific to a small set of proteins that are masked by the overall glycan analysis. In a rodent model, only certain glycoproteins were fucosylated in serum, whereas most all glycoproteins were fucosylated in bile (38). Preliminary data suggest that certain kinds of fucosylated proteins were selectively secreted into bile ducts but not into the conditioned medium in HepG2 cells (Nakagawa and colleagues, manuscript in preparation). These possibilities remain to be tested through the glycan analysis of specific proteins following resolution via 2-dimensional electrophoresis.

One change that was observed in HCC tissue was an increase in tetra-antennary *N*-linked glycan. Tetra-antennary *N*-linked glycans arise from the action of the enzyme GnT-V (16), which catalyzes the addition of  $\beta$ -1,6-GlcNAc to the growing *N*-linked glycan to form tri- and tetra-antenna-like oligosaccharides. Increased branching is associated with metastasis and has been associated with alterations in the hexosamine cycle and activation of the AKT pathway (19, 39–42). It has also been reported that expression of GnT-V in the liver was dramatically enhanced in the hepatocarcinogenesis of a rodent model. Increases in  $\beta$ -1,6-GlcNAc structure through upregulation of GnT-V have long been associated with cancers but this report represents the first oligosaccharide analyses with liver cancer tissue. The result was consistent with immunohistochemical analysis of GnT-V in HCC tissue (43) and is consistent with the hypothesis that the elevated expression of UDP-*N*-acetylglucosamine:  $\alpha$ -mannoside  $\beta$ -1,6 *N*-acetylglucosaminyltransferase (GnT-V) is an early event in hepatocarcinogenesis.

There are reports in the literature that increased branching of *N*-glycans correlates with reduced intercellular adhesion of epithelial cells (44). These published observations and our findings reported here lead us to propose the following working model (Fig. 5). We speculate that HCC is associated with an increase in tetra-antennary glycan addition to proteins involved cell–cell adhesion and/or tight junction integrity. The resulting weaker cell–cell junctions permit "leaking" of core fucosylated glycopro-



**Figure 5.** Proposed model. Left, in the normal liver, hepatocytes (white hexagons) surround the biliary space (black hexagons). Tight junctions present near the apical (biliary) surfaces of the hepatocytes isolate bile contents from basolateral contents (blood). Right, as HCC develops, the impermeable barrier weakens, permitting leakage of core fucosylated glycoproteins (black ovals) into the bloodstream.

teins from the biliary system, which are predominantly fucosylated (32), into the blood (and potentially other molecules that are bile specific). This model would account for the apparent discrepancy between core fucose levels present in the serum (and elevated in HCC) versus the tissue (nondifference between tumor and nontumor). Further characterization of the proteins that carry the tetra-antennary glycans will be required to test the model.

The identity of the proteins that contain tetra-antennary glycan are unclear but under investigation. It is understood that the level of tetra-antennary glycan was low in the HCC samples and represents only a minor species of the total glycan pool. This is not unexpected as conformationally, not all proteins will have the space to allow for a tetra-antennary glycan. Tetra-antennary glycans are usually observed on cell surface molecules, such as growth factor receptors and adhesion molecules. While these molecules are partially cleaved from the cell surface, it might be difficult to find them in circulation as they would represent only a minor fraction of serum glycoproteins. More advances in glycotecnology for oligosaccharide analysis may be required to fully characterize the *N*-glycan and to identify the proteins containing these alterations.

In conclusion, we have conducted *N*-linked glycan analysis of HCC and adjacent tissue in an effort to determine whether the increase in core fucosylation found on liver-derived serum proteins from patients with HCC was a result of increased fucosylation. Surprisingly, no increase in fucosylation was observed in HCC. In contrast, increased levels of branching, most notably, increased levels of tetra-antennary glycan were observed.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

**Conception and design:** A.S. Mehta, P. Norton, H. Liang, E. Miyoshi, T. Block

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