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associated with prognosis and recurrence in HCC than AFP or PIVKA-II are highly

desirable.

Glycosylation is one of the most common post-translational protein

modifications. Alterations in the N-glycosylation profiles of glycoproteins have been

suggested to play important roles in the proliferation, differentiation, invasion and

metastasis of malignant cells. Glycan species can be analyzed and characterized

using mass spectrometry and the profiling of these molecules when they are secreted

or shed from cancer cells is also performed. Hence, some glycoproteins have been

suggested as biomarkers of human carcinomas such as ovarian cancer, breast

cancer and HCC(16-19). Of note, changes to the N-linked glycan modification of

glycoproteins occur during the tumorigenesis and progression of HCC lesions.

However, the correlation between the N-glycan profile and tumor-associated

characteristics such as the degree of malignancy and prognosis has not been

previously evaluated in HCC. Recently, we developed a novel glycomics method that

facilitates high-throughput and large scale glycome analysis using an automated

glycan purification system, SweetBlot. This approach enables us to profile serum

N-glycans quantitatively. Using this quantitative N-glycomics procedure via

glycoblotting technology, which is both highly accurate and can be conducted on a

large scale, we have previously evaluated the potential of using N-glycans as markers

of the prognosis and recurrence of HCC (20).

In our current study, we have evaluated preoperative blood samples from a

HCC patient cohort from which we purified serum $N$-glycans using our glycoblotting

method (21, 22). We performed $N$-glycan profiling using mass spectrometry to search

for factors related to prognosis and recurrence by analysis of patient outcomes in 369

consecutive HCC cases that had undergone a primary curative hepatectomy at our

medical facility. We sought through this screen to correlate $N$-glycan levels on

glycoproteins with the clinicopathologic characteristics and the outcomes of HCC.

## Methods

### Patients

Between April 1999 and March 2011, 369 consecutive adult patients

underwent a hepatectomy procedure for HCC at our center and this sample

population was examined in the current study. Patients with extrahepatic metastases

had been excluded from this cohort because the outcomes of a hepatectomy in these

cases are typically very poor. The mean age of the patients in the final study group

was 62.7 $\pm$ 10.6 years (range, 33-90), 301/369 (81.6%) cases were male, 176

(47.7%) were hepatitis B virus surface antigen-positive, 119 (32.2%) were hepatitis C

virus antibody-positive, and 120 (32.5%) were designated as F4 based on the New

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Inuyama Classification system (23). The preoperative serum AFP and PIVKA-II levels

were simultaneously measured in the patients using standard methods at least two

weeks before the hepatectomy at the time of the imaging studies. Among the 369

patients in the cohort, 358 (97.0%) were categorized as Child-Pugh class A. According

to the TNM stage revised by the Liver Study Group of Japan in 2010(24), 26 (7.0%)

patients were in stage I, 172(46.6%) in stage II, 111(30.1%) in stage III and 60(16.3%)

in stage IVA. The patients were followed up for a median of 60.7 months (range,

9.8–155.1). As a normal control group, 26 living related liver transplantation donors

were selected. They were evaluated of eligibility by for donors by liver function tests,

measurements of the tumor markers AFP and PIVKA-II, and also by X-ray

photographs of chest and abdomen and dynamic computed tomography. Their mean

age was 40.0 with a range of 20-48. Of 26 controls, 15 (57.7%) were male and 11

(42.3%) were female. All controls were Japanese and not infected by hepatitis B and C

virus. This study was approved by the Institutional Review Board of the Hokkaido

University, School of Advanced Medicine. Informed consent was obtained from each

patient in accordance with the Ethics Committees Guidelines for our institution.


**Experimental procedures**

**Serum *N*-glycomics via glycoblotting**

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*N*-glycans from serum samples were purified by glycoblotting using BlotGlycoH. These are commercially available synthetic polymer beads with high-density hydrazide groups (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). All procedures utilized the SweetBlot automated glycan purification system containing a 96 well plate platform (System Instruments Co. Ltd. Hachioji, Japan).

**Enzymatic degradation of serum *N*-glycans**

Each 10 μL serum sample aliquot was dissolved in 50 μl of a 106 mM solution of ammonium bicarbonate containing 12 mM 1,4-dithiothreitol and 0.06% 1-propanesulfonic acid, 2-hydroxyl-3-myristamido (Wako Pure chemical Industries Ltd., Osaka, Japan). After incubation at 60°C for 30 min, 123 mM iodoacetamide (10 μl) was added to the mixtures followed by incubation in the dark at room temperature to enable reductive alkylation. After 60 min, the mixture was treated with 200 U of trypsin (Sigma-Aldrich, St. Louis, MO) at 37 °C for 2 h, followed by heat-inactivation of the enzyme at 90°C for 10 min. After cooling to room temperature, the *N*-glycans were released from the tryptic glycopeptides by incubation with 325 U of PNGase F (New England BioLabs, Ipswich, MA) at 37°C for 6 h.

**_N_-glycan purification and modification by glycoblotting**

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Glycoblotting of sample mixtures containing whole serum *N*-glycans was

performed in accordance with previously described procedures. Commercially

available BlotGlyco H beads (500 µl) (10 mg/ml suspension; Sumitomo Bakelite Co.,

Tokyo, Japan) were aliquoted into the wells of a MultiScreen Solvinert hydrophilic

PTFE (polytetrafluoroethlene) 96-well filter plate (EMD Millipore Co., Billerica, MA).

After removal of the water using a vacuum pump, 20 µl of PNGase F-digested samples

were applied to the wells, followed by the addition of 180 µl of 2% acetic acid in

acetonitrile. The filter plate was then incubated at 80°C for 45 min to capture the

*N*-glycans onto the beads via a chemically stable and reversible hydrazone bond. The

beads were then washed using 200 µl of 2 M guanidine-HCl in 10 mM ammonium

bicarbonate, followed by washing with the same volume of water and of 1% triethyl

amine in methanol. Each washing step was performed twice. The *N*-glycan linked

beads were next incubated with 10% acetic anhydride in 1% triethyl amine in

methanol for 30 min at room temperature so that un-reacted hydrazide groups would

become capped by acetylation. After capping, the reaction solution was removed

under a vacuum and the beads were serially washed with 2 x 200 µl of 10 mM HCl,

1% triethyl amine in methanol and dioxane. This is a pre-treatment for sialic acid

modification. On-bead methyl esterification of carboxyl groups in the sialic acids was

carried out with 100 µl of 100 mM 3-methyl-1-*P*-tolyltriazene (Tokyo Chemical

Industry Co., Tokyo, JAPAN) in dioxane at 60°C for 90 min to dryness. After methyl

esterification of the more stable glycans,   the beads were serially washed in 200 µl of

dioxane, water, 1% triethyl amine in methanol, and water. The captured glycans were

then subjected to a *trans*-iminization reaction with BOA (O-benzylhydroxylamine)

(Tokyo Chemical Industry Co., Tokyo, JAPAN) reagent for 45 min at 80°C. After this

reaction, 150 µl of water was added to each well, followed by the recovery of

derivatized glycans under a vacuum.

## MALDI-TOF and TOF/TOF analysis

The *N*-glycans purified by glycoblotting were directly diluted with

α-cyano-4-hydroxycinnamic acid diethylamine salt (Sigma-Aldrich) as ionic liquid

matrices and spotted onto the MALDI target plate. The analytes were then subjected

to MALDI-TOF MS analysis using an Ultraflex time-of-flight mass spectrometer III

(Brucker Daltonics, Billerica, MA) in reflector, positive ion mode and typically

summing 1000 shots. The *N*-glycan peaks in the MALDI-TOF MS spectra were

selected using FlexAnalysis ver. 3 (Brucker Daltonics, Billerica, MA). The intensity of

the isotopic peak of each glycan was normalized using 40 µM of internal standard

(disialyloctasaccharide, Tokyo Chemical Industry Co., Tokyo, JAPAN) for each status,

and its concentration was calculated from a calibration curve using human serum

standards. The glycan structures were estimated using the GlycoMod Tool

(http://br.expasy.org/tools/glycomod/), so that our system could measure

quativetatively 67 *N*-glycans.

## Hepatectomy

Anatomical resection is defined as a resection in which lesion(s) are

completely removed on the basis of Couinaud's classification (segmentectomy,

sectionectomy, and hemihepatectomy or more) in patients with a tolerable functional

reserve. Non-anatomical partial, but complete resection was achieved in all of our

cases. R0 resections were performed whilst the resection surface was found to be

histologically free of HCC. The indocyanin green retention rate at 15 minutes was

measured in each case to evaluate the liver function reserve, regardless of the

presence or absence of cirrhosis.

## HCC recurrence

For the first two years after the hepatectomy procedure, the HCC patients in

our cohort were monitored every three months using liver function tests,

measurements of the tumor markers AFP and protein induced by PIVKA-II, and also

by ultrasonography and dynamic computed tomography. At two years post-surgery,

routine computed tomography was performed only once in 4 months. If recurrence

was suspected, both computed tomography and magnetic resonance imaging were

performed, and if necessary, computed tomography during angiography and bone

scintigraphy were undertaken. This enabled a precise diagnosis of the site, number,

size, and invasiveness of any recurrent lesions.

## Statistics

The specificity, the sensitivity, cut-off and AUC (area under the curve) values

of selected $N$-glycans are shown in Table 1. This ROC (receiver operating

characteristics) analysis was carried out using R version 2.12.1. The patient survival

(PS) and disease-free survival rates (DFS) were determined using the Kaplan-Meier

method and compared between groups by the log-rank test. Univariate analysis of

variables was also performed, and selected variables using Akaike's Information

Criterion (AIC) (25) were analyzed with the Cox proportional hazard model for

multivariate analysis. Statistical analyses were performed using standard tests ($X^2$,

$t$-test) where appropriate using StatView 5.0 for Windows (SAS Institute Inc., Cary,

NC). Significance was defined by a $P$ value of     $< 0.05$.

## Results

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**Profiling of human serum glycoforms and ROC analysis in HCC patients and normal controls**

N–glycan profiles of blood samples from our HCC cohort were obtained by MALDI-TOF MS analysis utilizing the high throughput features of the instrument. We thereby identified 67 N-glycans from which we selected molecules that showed statistical differences by ROC analysis between HCC and disease-free individuals (normal controls, NC) comprising living related liver transplantation donors. Glycans with an AUC value greater than 0.80 were selected for analysis (Table 1) and box plots for these selected molecules (14 in total) are shown in Figure 1. Clear differences in the distribution of these factors are evident between the NC and HCC patients. The cut-off values were determined using the maximum values for specificity plus sensitivity. G2890 was elevated more than cut-off value in 305 (82.7%) of HCC patients and G3560 in 261 (70.7%).

**Causes of death**

There were 115 deaths in total among our 369 HCC patient cohort (31.2%). The causes of death were as follows: HCC recurrence (n = 97; 84.3%), liver failure (n = 6; 5.2%), and other causes (n =12; 10.4%).

**Univariate analysis and multivariate analysis of overall patient and disease-free survival**

The overall PS rates at 1, 3 and 5 years in our HCC cohort were 88.8%, 76.4% and 67.6% respectively. The DFS values for this groups at 1, 3 and 5 years were 64.0%, 35.5% and 27.4% respectively. The 14 serum N-glycans which were highly specific for HCC were evaluated for 3-year recurrence free survival by ROC analysis to determine the cut-off values about these N-glycans. The patients were divided to 2 groups by these cut-off values. The PS and DFS measurements associated with the selected 14 selected *N*-glycans were evaluated by univariate analysis. The P values for the PS rates associated with G2890, G1708, G3195, G3560, G2114, G1809, G3341, G1362 and G3865 were all less than 0.05. The DFS P values for G2890, G1708, G3195, G3560, G3341, G1362 and G3865 were also less than 0.05 (Table2). When clinical and tumor associated factors were evaluated by univariate analysis, albumin, Child-Pugh classification, AFP, AFP-L3, PIVKA-II, tumor number, tumor size, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion, macroscopic vascular invasion and Stage were found to be significantly associated with the PS rate. When the same analysis was undertaken for the DFS rate by univariate analysis, albumin, indocyanin green retention rate at 15 minutes, Child-Pugh classification, AFP, PIVKA-II, tumor number, tumor size, differentiation,

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microscopic portal vein invasion, microscopic hepatic vein invasion, macroscopic

vascular invasion, Stage and non-cancerous liver were found to be significantly

associated with this measure (Table 3) 5 .

The variable selection from 19 clinical and tumor associated factors in Table

3 and the 14 serum N-glycans using Akaike's Information Criterion (AIC) was

performed, and the selected valuables were analyzed with PS and DFS by multivariate

analysis. G3560 were found to be independent risk factors for PS (Tables 4) and

G2890 for DFS (Tables 5).

The PS rates of HCC cases with low serum G3560 levels at 5 years were

80.5% and of high serum G3560 at 5 years were 40.4%. The DFS outcomes

associated with low and high serum G2890 levels at 5 years were 21.3% and 35.1%,

respectively (Fig. 2).

**Relationship between clinical and tumor-associated factors in HCC and specific**

**glycans**

Among the low and high G2890 HCC groups, there were significant

differences found in a number of clinical and tumor-associated factors including

albumin, Child-Pugh classification, AFP, PIVKA-II, tumor number, tumor size,

microscopic portal vein invasion, microscopic hepatic vein invasion, macroscopic

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vascular invasion and Stage (Table 6). In comparing the low and high G3560 HCC

patients, significant differences were found in albumin, Child-Pugh Classification,

operative procedures, AFP, AFP-L3, PIVKA-II, tumor number, tumor size,

differentiation profiles, microscopic portal vein invasion, microscopic hepatic vein

invasion, macroscopic vascular invasion and Stage (Table 6).

## Discussion

The N–glycan profiles of a large cohort of HCC patients were obtained in our

current study by MALDI-TOF MS analysis and 67 of these molecules were thereby

quantified. Of this group of factors, 14 N-glycans showed higher relative peaks in the

HCC patients compared with normal controls and were chosen for further analysis.

These selected molecules were assessed for any correlation with surgical outcomes in

the HCC cohort (i.e. prognosis and recurrence) by univariate and multivariate

analysis. G3560 N-glycan was found to be significant prognostic factor and G2890

N-glycan was found to be significant recurrence factor for this disease. Moreover,

G2890 and G3560 were found to strongly correlate with a number of well-known

tumor-related prognostic and recurrent factors. These results show that quantitative

glycoblotting based on whole serum N-glycan profiling is a potent screening approach

for novel HCC biomarkers, and that the G3560 and G2890 N-glycans are promising

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biomarkers of the PS, DFS, and malignant behavior characteristics of HCC after a

hepatectomy.

Although glycans, once released from glycoproteins or glycopeptides, have

been subjected to fluorescent labeling and purification for detection by high

performance liquid chromatography (HPLC) previously, this method is time

consuming and therefore not suited to clinical diagnosis. Our novel analytical method

which we refer to as glycoblotting is far more rapid and accurate as evidenced by the

number of *N*-glycans detected in our current analysis. This chemoselective glycan

enrichment technology known as glycoblotting was developed in our laboratory to

purify oligosaccharides derived from glycoproteins in an effective and quantitative

manner, thus enabling serum glycan profiling via a simpler method (20). Our method

is also applicable to the fully automated analysis of multiple samples simultaneously.

It readily combines the isolation and labeling of oligosaccharides which can then be

subjected to conventional analytical methods including mass spectrometry. We had

already achieved high-speed quantitative and qualitative profiling of glycan

expression patterns in biological materials using this technology. In our present

study, we improved the method to allow quantitative analysis of high reproducibility

and accuracy using a calibration curve of human serum standards.   The analysis of

obtained 67 glycans profile was performed using this new developed technology. The

effectiveness of our method is evidenced by the identification of the G2890 and

G3560 *N*-glycans as highly promising clinical markers of HCC associated with the PS,

DFS and tumor malignancy rates of these cancers.

It has been reported that AFP is the most significant tumor marker and

independent predictor of prognosis for HCC (26), even in patients who have received a

hepatectomy (27). Although high levels of AFP in cases of fully developed HCC, or in

the serum of the host, are known to be associated with more aggressive behavior, and

increased anaplasis (28), AFP can also cause apoptosis in tumor cells (29). Moreover,

it has been suggested that AFP regulates the immune response and induces either

stimulatory or inhibitory growth activity (30). On the other hand, it is well known that

AFP may increase in some patients with acute and chronic hepatitis without HCC (31,

32), and that the elevation of AFP correlates with inflammation of background disease

and hepatocyte regeneration (33). Hence, because the AFP profile does not always

directly reflect the extent of tumor malignancy, the AFP levels do not influence patient

survival and recurrence. On the other hand, AFP and many important tumor markers,

such as carcinoembryonic antigen, carbohydrate antigen 125 and carbohydrate

antigen 19-9, are glycoproteins, and this means that the glycan profiles in serum are

altered by the onset of cancer. Indeed, the profiling of serum glycans has been

performed previously as a screen for distinct potential glycan biomarkers of ovarian

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cancer and breast cancer (18, 19). Hence, we surmised that highly specific

glycoprotein markers of HCC should be detected by monitoring the serum

glycosylation profile in these patients. In the view point of glycan structure, both

G2890 and G3560 are multiply branched (G2890 is tri-antennary and G3560 is

tetra-antennary) glycans with a core fucose. In addition both glycans have one

non-sialylated branch, i.e., G2890 and G3560 are tri-antennary di-sialylated glycan

and tetra-antennary tri-sialylated glycan, respectively. The structure of G2890 and

G3560 is quite different from the AFC-L3 (core fucosylated bi-antennary glycan) and

CA19-9 (sialylated lewis (a) antigen), which are the well-known biomarker related to

HCC except for the core fucosylation.

There have been several previous studies of glycans in HCC. Kudo et al

reported that *N*-glycan alterations are associated with drug resistance in HCC in vitro

(34). In other reported clinical studies, only specific glycans have been assessed in

relation to HCC. Vanhooren et al were the first to analyze the function of HCC-specific

glycans, and reported that a triantennary glycan (NA-3Fb) correlated with the tumor

stage and AFP levels in HCC patients (17). However, this study analyzed 44 patients

with HCC but did not evaluate relationship between the *N*-glycans and the clinical

and pathological factors of this disease, the clinical course after hepatectomy, or

prognosis and recurrence. In our current study in contrast, we analyzed a far larger

cohort than any other previous report, and evaluated a comprehensive panel of

clinical and pathological parameters in relation to the N-glycan profile in HCC. Tang

et al also described some HCC-specific glycans in their previous study (35) which we

did not find to be significant in our current analyses. This is likely due to the fact that

the patient number in their study was smaller than ours, and the fact that the N

glycome profile in serum is gender and age dependent (36). In this study, the mean

age and the distribution of gender and infection of hepatitis B and C virus were

difference between NC and HCC patients. However, the selected 14 serum N-glycans

were quantified by our MALDI-TOF MS analysis and compared with NC by ROC

analysis. These were statistically different between HCC and NC with respect to the

quantity. Because these 14 serum N-glycan of which the AUC values were greater

than 0.80 were revealed to be specific for HCC, they had a high discriminating ability

to differentiate HCC from NC. Further analyses are required to determine whether

G2890 and G3560 are elevated in patients with hepatitis B, hepatitis C and/or

cirrhosis without hepatocellular carcinoma.

The most important adverse prognostic factor for liver resection and

transplantation in HCC has been found to be microscopic venous invasion(5).

However, microscopic portal invasion is not diagnosed preoperatively, and is revealed

only by pathological examination. New biomarkers which are more strongly

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associated with prognosis and recurrence of HCC than AFP, AFP-L3 or PIVKA-II are

therefore highly desirable. Our current data show that the *N*-glycans G2890 and

G3560 correlate closely with well-known tumor-related prognostic and recurrent

factors such as tumor number, size, microscopic portal vein invasion, microscopic

hepatic vein invasion, differentiation, macroscopic vascular invasion, Stage, AFP,

AFP-L3, and PIVKA-II (Table 6). Moreover, when G2890 and G3560 were

simultaneously included in multivariate analysis for PS and DFS with AFP, AFPL3

and PIVKA-II, p-values of G2890 and G3560 were lower than AFP, and AFPL3 and

PIVKA-II were not selected as valuables by AIC. We demonstrate that these are novel

independent prognostic factors for HCC that are related to the survival and

recurrence of this disease and that show a lower P-value than other established

tumor factors. Hence, we predict that G2890 and G3560 will prove to be markers that

can preoperatively predict HCC tumor malignancy including microscopic portal vein

invasion, and the PS and DFS rates more accurately and with more potency than the

more well-known biomarkers.

## Acknowledgments

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## Figure legends

**Figure 1.** Box plots of the disease-free individuals (NC) and HCC patients for the selected 14 *N*-glycans. The dotted lines in the graphs represent the cut-off values determined in this analysis. These graphs were drawn using R version 2.12.1.

**Figure 2.**

The PS rates of HCC cases with low and high serum G3560 levels at 5 years were 80.5% and 40.4% respectively. The DFS outcomes associated with low and high serum G2890 levels at 5 years were 21.3% and 35.1%, respectively.

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