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

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**

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

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,

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

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

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

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

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.

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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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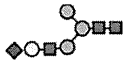
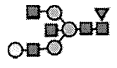
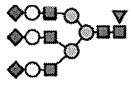
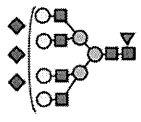
N-glycans	m/z		specificity (%)	sensitivity (%)	cut-off value	AUC
G2032	2032.724		100	86.45	1.115	0.968
G2890	2890.052		92.31	82.66	0.844	0.91
G1793	1793.672		92.31	75.61	1.963	0.9
G1708	1708.619		88.46	77.51	0.604	0.896
G1870	1870.672		88.46	75.88	2.886	0.873
G1955	1955.724		100	59.89	3.913	0.873
G3195	3195.163		92.31	71.27	6.109	0.864
G3560	3560.295		88.46	71.27	0.091	0.851